The Role of FPR1 and GPR32 in Human Inflammation

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Summary

Inflammation is the natural reaction of the body toward tissue injury or pathogen invasion with the ultimate goal to restore homeostasis. When tissue resident APCs sense a perturbation, they release an array of chemokines and signalling molecules, which in turn attract further leukocytes into the affected tissue. Neutrophils, highly specialized microbial killers, are the first cells attracted from the blood stream to counter the noxious agents. In second place, the activated environment also promotes the development of classically activated M1 macrophages in the tissue, which work in concomitance with neutropihls and sustain the inflammatory reaction.

During this pro-inflammatory phase of inflammation, FPR1, a receptor expressed by mammalian phagocytic leukocytes, plays an important role in chemotaxis, killing of microorganisms through phagocytosis, and the generation of reactive oxygen species. We show here that primary human neutrophils, monocytes and resting macrophages do express the receptor on their cell surface. Polarization of primary human macrophages toward the pro-inflammatory M1 phenotype further increases FPR1 mRNA levels but did not consistently increase protein expression or chemotaxis towards the FPR1 ligand fMLF. In contrast, M2 macrophages, which are associated with the resolution phase of inflammation, have reduced FPR1 cell surface expression and are not chemotactic towards fMLF, limiting the pro-inflammatory role of FPR1 to chemotaxis and superoxide production in resting and pro-inflammatory M1 macrophages.

Once the invading pathogens have been contained, this pro-inflammatory cascade has to be stopped, and resolution programs need to be initiated in order to restore tissue homeostasis. Two key cellular events characterize the resolution of inflammation: a reduction in neutrophil infiltration and a re-polarization of the M1 macrophages toward a pro-resolution “M2-like” phenotype.

Lipid mediators play a major role in the resolution of inflammation and we characterized the mechanisms of action and receptor triggering of one of these specialized pro-resolution mediators, RvD1, on primary human macrophages. We show that the RvD1 receptor GPR32 is present on resting, pro-inflammatory M1 and alternatively activated primary human M2 macrophages. Accordingly, stimulation of resting primary human macrophages with 10nM RvD1 for 48h reduces the secretion of the pro-inflammatory cytokines IL-1β and IL-8, abolishes chemotaxis to several chemoattractants like chemerin, fMLF, and MCP-1, and doubles the phagocytic activity of these macrophages towards microbial particles. In contrast,

these functional changes are not accompanied by surface expression of markers specific for alternatively activated M2 macrophages. Similar pro-resolution effects of RvD1 are observed when pro-inflammatory M1 macrophages are treated with RvD1. In addition, we show that these RvD1 mediated effects are GPR32 dependent because reduction of GPR32 expression by siRNA, ablates these pro-resolution effects in primary human macrophages. Taken together our results indicate that in humans RvD1 triggers GPR32 to polarize and re-polarize macrophages towards a pro-resolution phenotype, supporting the role of this mediator and GPR32 in the resolution of inflammation in humans.

To conclude, macrophage polarization is essential for the correct function of the inflammatory cascade and inflammatory diseases are frequently associated with a lack in the dynamic changes of macrophage polarization. In this context, our studies indicate that antagonistic blocking of FPR1 or triggering of GPR32 with RvD1 mimetics may be beneficial in human inflammatory diseases, preventing further leukocyte infiltration and promoting the resolution of inflammation.

Zusammenfassung

Die Entzündung ist die natürliche Reaktion des Körpers auf eine Gewebeverletzung oder auf das Eindringen von Pathogenen mit dem Ziel, die Homöostase aufrechtzuerhalten. Wenn gewebegebundene APCs eine Störung erkennen, setzen sie eine Reihe von Chemokinen und Signalmolekülen frei, welche wiederum weitere Leukozyten zum betroffenen Gewebe locken. Neutrophile sind die ersten Zellen, welche aus der Blutbahn ins Gewebe angelockt werden, um das schädliche Agens anzugreifen. Zweitens fördert die betroffene Stelle die Entstehung von aktivierten M1-Makrophagen im Gewebe, welche zusammen mit den Neutrophilen die inflammatorische Reaktion verstärken.

Während dieser pro-inflammatorischen Phase der Entzündung, FPR1, ein exprimierter Rezeptor auf den phagozytierenden Leukozyten, spielt eine grosse Rolle in der Chemotaxis, dem Abtöten von Mikroorganismen durch Phagozytose und der Generierung von Sauerstoffradikalen. Hiermit zeigen wir, dass dieser Rezeptor auf den Oberflächen der primären humanen Neutrophilen, Monozyten und ruhenden Makrophagen exprimiert wird.

Die Polarisation der primären humanen Makrophagen gegen den pro-inflammatorischen M1-Phänotyp erhöht zwar das FPR1-mRNA-Level, erhöht allerdings nicht andauernd die Proteinexpression oder Chemotaxis gegen den FPR1-Liganden fMLF. Im Gegensatz dazu reduzieren M2-Makrophagen, welche mit der Auflösungsphase der Entzündung assoziiert sind, die FPR1-Zelloberflächenexpression und sind nicht chemotaktisch gegen fMLF. Deshalb ist die pro-inflammatorische Rolle des FPR-1 bezüglich der Chemotaxis und der Sauerstoffradikalproduktion nur auf ruhende und M1-Makrophagen beschränkt.

Sind die eingedrungenen Pathogenen eingeschlossen, muss die pro-inflammatorische Kaskade gestoppt werden und die Resolutionsprogramme müssen initialisiert werden, um die Gewebehomöostase wieder herzustellen. Zwei Schlüsselereignisse auf der zellulären Ebene charakterisieren die Auflösung der Entzündung: eine Reduktion der Neutrophileninfiltration und die Repolarisation der M1-Makrophagen in einen „M2-ähnlichen“-Phänotyp.

Lipidmediatoren spielen eine wichtige Rolle in der Auflösung der Entzündung und wir haben den Aktionsmechanismus und die Rezeptorauslösung einer dieser spezialisierten pro-resolutions-Mediatoren, RvD1, auf primären humanen Makrophagen charakterisiert. Wir zeigen auf, dass der RvD1-Rezeptor GPR32 auf ruhenden, pro-inflammatorischen M1 und alternativ aktivierten primären humanen M2-Makrophagen vorhanden ist. Dementsprechend reduziert die Stimulation der primären humanen Makrophagen mit 10nM RvD1 für 48 Stunden die Sekretion der pro-inflammatorischen Zytokinen IL-1β und IL-8, hebt die

Chemotaxis durch Chemerin, fMLF und MCP-1 auf und verdoppelt die Phagozytoseaktivität dieser Makrophagen gegen mikrobielle Partikel. Im Gegensatz dazu werden diese funktionellen Veränderungen nicht mit einer Oberflächenexpression von spezifischen Markern für die alternativ aktivierten M2-Makrophagen begleitet. Ähnliche auflösende Effekte des RvD1 werden bei der Behandlung von pro-inflammatorischen M1-Makrophagen mit RvD1 beobachtet. Zusätzlich zeigen wir auf, dass die RvD1-vermittelten Effekte GPR32-abhängig sind, da die Reduktion von GPR32, welches durch siRNA erzeugt wird, diese pro-resolution-Effekte in den humanen Makrophagen stoppt. Zusammengefasst zeigen unsere Resultate, dass RvD1 GPR32 aktiviert, was zu einer Polarisierung und Repolarisierung der Makrophagen gegen einen pro-resolutions-Phänotyp führt, was wiederum die Rolle dieses Mediators und GPR32 in der Auflösung der Entzündung bei Menschen unterstützt.

Die Makrophagenpolarisation ist für die korrekte Funktion der inflammatorischen Kaskade unerlässlich. Ausserdem sind inflammatorische Krankheiten häufig mit einem Fehlen der dynamischen Veränderungen dieser assoziiert. In diesem Kontext zeigen unsere Studien, dass das antagonistische Blocken von FPR1 oder dem Auslösen von GPR32 mittels RvD1 einen vorteilhaften Einfluss auf humane inflammatorische Krankheiten haben könnten, da sie eine weitere Leukozyteninfiltration verhindern und die Auflösung der Entzündung fördern.

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List of abbreviations

AA: arachidonic acid

APC: antigen presenting cells

AT: aspirin triggered

COX: cyclooxygenase

CYP: cytochrome P450 monooxygenase pathway

DAMPs: damage associated molecular pattern molecules

DCs: dendritic cells

DHA: docosahexanoic acid

EPA: eicosapentaenoic acid

fMLF: formyl-methionyl-leucyl-phenylalanine peptide

FPR: formyl peptide receptor

GPCR: G protein-coupled receptor

HETE: hydroxy-eicosatetraenoic acid

HIV: human immuno-deficiency virus

IBD: inflammatory bowel disease

IFN: interferon

LOX: lipoxigenase

LPS: lipopolysaccharide

LTB4: leukotriene B4

LXA4: lipoxin A4

MCP-1: monocyte chemoattractant protein 1

NETs: neutrophil extracellular traps

PAMPs: pathogen associated molecular pattern molecules

PDCD4: programmed cell death protein 4

PG: prostaglandin

PLA2: phospholipase 2

PPRs: pattern recognition receptors

PUFA: polyunsaturated fatty acids

RNS: reactive nitrogen species

ROS: reactive oxygen species

RvD1: resolving D1

RvE1: resolving E1

TLR: toll-like receptor

TRPA1: transient receptor potential cation channel A1

T2D: type 2 diabetes

1. Introduction

1.1 Inflammation

1.1.1 Acute and chronic inflammation

Inflammation is the natural occurring response of the body against harmful stimuli such as infection, traumatism and irritation. Its aim is the elimination of pathogens, removal of damaged tissue and ultimately restoration of homeostasis [1]. The physiology of this process begun to elicit interest since the days of Celsus (about 30 BC to 38 AD), who described inflammation in terms of cardinal signs: heat, redness, swelling and pain [2]. In 1794, the Scottish surgeon John Hunter first realized that inflammation was not a disease itself, but rather a beneficial process that evolved as an adaptive response to some violence or some disease [3]. Although later innumerable individuals described many of the mechanisms associated with tissue injury and inflammation, the chemical mediators that bring about these events were only discovered in the last 40 years of research [4].

Acute inflammation lasts from few hours to few days and involves the activation of the innate immune response and later of the adaptive immune response. The inflammatory signs described by Celsus can be explained today by increases in blood flow, activation of the plasma cascade and augmented permeability of blood vessels. This process is coupled with a quick migration of leukocytes from the bloodstream to the injured site, where the inciting agent is neutralized. After completing their job, leukocytes need to be cleared from the inflamed site in order to restore tissue homeostasis [5]. There are, however, scenarios where inflammation can become uncontrolled and lead to a pathological state lasting months or even years, known as chronic inflammation. Whereas the events that are involved in the acute inflammatory response following infection or tissue injury are well understood, much less is known about the causes and mechanisms of systemic chronic inflammation, which underlies a variety of diseases, including cardiovascular disease and type 2 diabetes (T2D). One of the causes leading to chronic inflammation is a failure of the resolution of inflammation, once incorrectly thought to be a mere passive process [6]. Today it is widely accepted that resolution of inflammation is a highly controlled and active process, which is controlled by endogenous pro-resolution mediators [7, 8], and resolution physiology has elicited much interest since then.

1.1.2 Onset of inflammation and leukocyte infiltration

When there is a perturbation of tissue homeostasis caused by tissue injury or pathogen invasion, the innate immune system responds in a first line with the plasma cascade and a concerted action of professional phagocytes: neutrophils, monocytes, macrophages, and dendritic cells (DCs). Pathogen associated molecular pattern molecules (PAMPs) and damage associated molecular pattern molecules (DAMPs) are recognized by tissue resident antigen presenting cells (APCs) via pattern recognition receptors (PPRs) [9]. Activated APCs then secrete large amounts of cytokines and signalling molecules which in turn attract further leukocytes into the affected tissue from the blood stream.

The first type of white blood cells recruited to the chemotactic milieu created by APCs is the neutrophil. Recruitment of these first key role players is driven by chemotactic agents such as the complement components C3a and C5a, the lipid mediator leukotriene B4 (LTB4), platelet activating factor, and CXCL8 also known as IL-8 [10]. The effects of APC-derived chemokines and lipid mediators in promoting neutrophil infiltration have been extensively studied in vivo in the pleural cavity, the peritoneum and the lung [11-15]. As a front-line defenders, neutrophils are highly specialized in killing pathogens by three main mechanisms: phagocytosis, release of anti-microbial peptides and generation of neutrophil extracellular traps (NETs) [16]. Neutrophils can internalize many types of microbes and digest them in the phagosomes into which hydrolytic enzymes, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are secreted. Additionally they secrete a variety of anti-microbial peptides in a process called degranulation [17], and use NETs, a web of fibers composed of chromatin and serine proteases that trap and kill pathogens in the extra cellular space[18]. Taken together neutrophils are highly proficient microbial killers, they have, however, a short life span and need to be cleared from the tissue once the noxious agents have been eliminated to constrain the tissue damage caused by ROS, NOS, granules and apoptotic cells.

The second cell type which appears during the course of inflammation is the monocyte. At least three different monocyte subtypes have been characterized in human blood [19]. The classical type, which represent the majority of circulating monocytes, express high CD14 and low CD16 ($CD14^{high}CD16^{low}$). The remaining monocytes express CD16 but differ in the expression level of CD14. $CD14^{low}CD16^{high}$ monocytes have been shown to produce high levels of TNFα upon LPS stimulation [20], while $CD14^{high}CD16^{high}$ are the least characterized and may represent a transitional phenotype which produces IL-10 [21]. There is evidence that neutrophils both produce [22] and modify [23] chemokines to create a milieu that favours the infiltration of $CD14^{high}CD16^{low}$ classical monocytes. It is thought that activation of different

G protein-coupled receptors (GPCRs), in particular of the formyl-peptide receptor family (FPRs), plays a fundamental role at this stage of inflammation, controlling chemotaxis and phagocytosis [24]. For example, formylated peptides bind FPR1 inducing classical monocyte chemotaxis [25], while the lipid mediator Lipoxin A4 (LXA4) binds FPR2/ALX and recruits non-phlogistic monocytes, which exert higher phagocytosis [26, 27]. After adhesion to the vascular endothelium, monocytes extravasate utilizing the interaction between their β1 integrins with the endothelial-expressed E-selectins [10] and once in the tissue, differentiate to macrophages, dendritic cells or osteoclasts, depending on the cytokine milieu [28].

1.1.3 Macrophage plasticity and polarization

Macrophages are not mere phagocytic cells, but rather true master regulators of inflammation with key roles during the whole inflammatory response, from initiation to resolution [29, 30]. Moreover they fulfil other homeostatic functions beyond defence against harmful stimuli such as tissue remodelling in ontogenesis and regulation of metabolic functions [31-33]. It is therefore not surprising that macrophages are one of the most plastic and heterogeneous cells in the immune system. In tissue they respond to environmental stimuli with polarization to different functional phenotypes ranging from the pro-inflammatory M1 to the alternatively activated M2, which play different roles in health and disease [34]. The canonical IRF/STAT1 and NF-κB signalling pathways activated by interferons (IFNs) and TLR signalling drive macrophages toward a M1 phenotype. This polarization state is characterized by expression of high amounts of pro-inflammatory cytokines, production of ROS and RNS, and strong microbicidal activity [35]. On the other side of the spectrum M2 macrophages are driven by the Th2 cytokines IL-4 and IL-13. The IL-4 type I and type II receptor activation induces STAT6 which in turn regulates the transcription of typical M2 polarization genes such as the mannose receptor (CD206), resistin-like α, and chitinase 3-like 3 [32, 36, 37]. This polarization state is paired with proficient phagocytosis, high expression of scavenging molecules, the expression of CD206, and high production of IL-10 and IL-1ra [32, 38]. While M1 macrophages are known to play an important role in the initiation and progression of inflammation, the M2 polarization state is associated with the Th2 response to parasite infections, wound healing, tissue repair, and restoration of homeostasis [35].

Importantly, M1 and M2 are only the extremes of a continuum of activation states and intermediate phenotypes in specific stages of inflammation such as the resolution phase of inflammation have been identified [39]. In this context, inflammatory diseases are frequently associated with a lack in the dynamic changes of macrophage polarization, with M1

macrophages implicated in sustaining inflammation and M2 or M2-like macrophages associated with resolution or dampening of chronic inflammation [40]. Given the fact that the polarization state can, to some extent, be reversed in vitro and in vivo [41-44], specific therapies targeting macrophages and their polarization in different inflammatory conditions are now taking the first steps into clinical trials [35].

1.1.4 Pro-inflammatory signals and chemotaxis

The phagocytic partnership taking place during inflammation is a highly coordinated process regulated by a complex network of cytokines and lipid mediators. Fine-tuning of the expression of cytokines, lipid mediators and their respective receptors plays a fundamental role during the different stages of inflammation.

Being the messengers of the immune system, cytokines have to cover very different functions. Classical pro-inflammatory cytokines such as IL-1β and TNFα are potent inducers of the NF-κB pathway and of the MAP kinase cascade [45, 46]. In first place these cytokines act locally by increasing the vascular inflammation and by promoting the formation of high endothelial venules and chemotaxis of leukocytes in the affected loci [47]. Additionally, they also act in a systemic way inducing production of acute phase proteins from the liver and consequently complement activation [48]. Other cytokines work only locally, like interferons (IFNs), which induce an anti-viral effect through the JAK/STAT pathway in infected cells, or through the Th1 promoting IL-12, which works at the interface between innate and adaptive immunity [49].

Directed chemotaxis of nearby responsive cells can be induced by a family of small cytokines named chemokines, by some lipid mediators and by bacterial products like formylated petides. Inflammatory chemokines and lipid mediators are produced at high concentrations by leukocytes, endothelial and epithelial cells during specific stages of inflammation and determine the migration of specific leukocytes to the inflamed area.

During the early stages of acute inflammation, resident immune cells expressing a wide variety of PRRs recognize pathogens and respond by releasing a variety of chemokines. Activated tissue macrophages and DCs release high amounts of CXCL1, CXCL2, CCL2 (MCP-1), IL-8, other chemokines [50], and the lipid mediator LTB4 [51]. Another source of chemoattractants is the endothelium activated by IL-1β and TNFα , which produces a plethora of chemokines, including CCL2, CCL3, CCL4, CCL5 (RANTES), CXCL1, CXCL2, CXCL3, CXCL5, and IL-8 [52]. Additionally the activity of some chemokines like CXCL1 and IL-8 can be further increased by cleavage through metalloproteinases released from neutrophil

granules [29]. During this initial phase of inflammation CXCL1, CXCL2 and IL-8 mediate adhesion and transmigration of neutrophils. After the transmigration, several chemoattractant gradients, such as LTB_4 [53], CXCL1 and CXCL2 [54], guide neutrophils as they move through the interstitium to the acute inflammatory source, where they act as terminal effector cells, secreting granules and phagocytosing particles.

The activated endothelium also attracts monocytes through the secretion of MCP-1, which binds to its cognate receptor CCR2 and is important for the normal inflammatory monocyte migration into the tissue [55]. However, when the noxious agents have been countered, resolution of inflammation starts and a rapid change in the chemoattactant milieu takes place. Increased expression of CXCR5 on apoptotic neutrophils leads for example to the sequestration of secreted CXCL3 and CXCL5 dampening further chemotaxis of neutrophils and monocytes [56]. During this phase of inflammation a series of specialized pro-resolution lipid mediators further regulate leukocyte chemotaxis. LXA4 for example is known to recruit non-phlogistic monocytes into the tissue, which are bound to develop to pro-resolution macrophages [26, 27], while resolvin E1 (RvE1) and resolvin D1 (RvD1) block neutrophil chemotaxis via the BLT1 [57] and the FPR2/ALX [58] receptors, respectively, initiating the resolution of inflammation and tissue homeostasis.

1.1.5 G protein coupled receptors

The first evidence that chemoattactants bind and activate GPCRs was noted from the fact that pertussis toxin could alter the binding affinity of formylated peptides [59]. Later, the first chemoattractant receptors were identified through molecular cloning and analysis of their deduced protein sequence, namely the receptors for fMLF [60] and C5a [61]. Following the initial cloning efforts, other well known chemoattractant receptors such as BLT1 (receptor for LTB4) were identified as GPCRs [62].

GPCRs are the largest protein superfamily in the mammalian genomes, which shares a common seven trans-membrane topology. They mediate many cellular responses to different extracellular signals ranging from photons and small molecules to peptides and proteins [63]. GPCRs play also a pivotal role in inflammation, from cell chemotaxis to vascular endothelial permeability and regulation of inflammatory gene transcription. Notably they are abundantly expressed by neutrophils, monocytes and macrophages [64].

Signalling by GPCRs is initiated by binding of a specific ligand, which induces a conformational change in the receptor. The majority of signalling is dependent on G-proteins, however β-arrestins also play a role [65]. In the G-protein dependent pathway, the activated

receptor can exchange its bound GDP for GTP, allowing the dissociation of the α subunit from the βγ subunit. Depending on the type of α subunit (Gαs, Gαi, Gαq/11 or Gα12/13) different transduction pathways are possible [66]. Major downstream effectors and signalling molecules include Ca^{2+}, cAMP, protein kinases, lipid kinases, lipases, GTPases and transcription factors [64]. The agonist induced signalling can induce several physiological effects such as chemotaxis [67], granule release, and activation of several protein kinases [68, 69]. Some GPCRs bind molecules such as C5a which leads to the production of ROS [70, 71], while other ligand-GPCR duos are involved in inflammatory pain and endothelial permeability, like bradykinin and its corresponding receptors B1 and B2 [72]. Even more importantly GPCRs give a great contribution to the regulation of inflammatory gene expression, activating transcription factors such as CREB, c-Jun, NF-κB and STAT3 [73]. Particularly NF-κB activation, a pivotal transcription factor in inflammation, has been shown to be regulated by GPCRs via either the Gα/Gβγ-dependent pathway [74] or the β-arrestin pathway [75]. β-arrestins are adapter proteins that form complexes with a GPCR upon ligand binding and phosphorylation of the receptor. They function as alternative signal transducers forming complexes with components of the ERK1/2 and JNK3 MAP kinase cascades and have a pivotal role in the desensitisation of GPCRs [76].

Being essential for the inflammatory response as well as for other important physiological functions, GPCRs have become interesting targets for therapeutic intervention during the last years [77, 78].

1.1.6 The Formyl peptide receptor 1 (FPR1)

About forty years ago, it was shown that small N-formylated peptides produced by the catabolism of either bacterial products [79, 80] or by disrupted mitochondria [81] are potent activators of neutrophils. These peptides engage a specific class of GPCRs, named formylated peptide receptors, and trigger neutrophil responses such as chemotaxis, release of enzymatic granules and ROS production [82].

FPR1, which binds N-formylated peptides from bacteria with high affinity, was the first neutrophil chemoattractant receptor to be characterized biochemically [69] and has been shown to be a key player in innate immunity and host defence [24, 25, 81]. In the past few years, an array of endogenous and microbial peptides with a wide range of structures, have been shown to activate FPR1, which is nowadays seen as a promiscuous receptor [25]. Other than formylated peptides derived from bacteria, several peptides of the HIV-1 envelope proteins, like gp41, have been shown to activate myeloid cells via FPR1 [83]. In addition,

host-derived agonists of FPR1 include the antimicrobial neutrophil granule protein cathepsin G, which recruits DCs and thereby links innate with adaptive immunity [84].

The abundance of ligand binding to FPR1 and the different effects seen upon its activation indicate that several signalling pathways can be triggered upon FPR1 stimulation. Chemoattractant receptors, including FPR1, are coupled to the heterodimeric protein of the Gαi subtype. Upon activation, a conformational change allows the interaction with the G protein, triggering the exchange of GDP to GTP in the G protein α subunit and the dissociation of the βγ subunit [85]. Chemotaxis toward pathogens is initiated by both the activated Gα and the Gβγ subunits through the activation of phosphoinositide 3-kinase γ (PI3Kγ) [86, 87]. Other functional effects, like for example ROS production, are mediated by other signalling pathways and are dependent on Rac [88] and PKC [89] signalling. Another important feature of the receptor is its desensitization upon ligand stimulation, preventing further stimulation [90]. When FPR1 is activated by a cognate ligand it gets phosphorylated by GPCR kinases, inducing the linking of arrestin molecules, which prevent further binding to G proteins, leading to its inactivation and internalization [91, 92].

The important inflammatory actions initiated by the activation of FPR1, such as chemotaxis, ROS production and granule release, are underscored by its high expression in myeloid cells like neutrophils, monocytes and macrophages [93]. In fact, the expression of FPR1 is limited to the myeloid cell linage since PU.1, a transcription factor necessary for its transcription, is myeloid specific [94]. Despite the large knowledge on the expression and function of FPR1 in neutrophils, much less is known about its expression and function on differentially polarized macrophages. This object of interest will be therefore discussed in more details in the result section, in the manuscript "Regulation of the Formy Peptide Receptor 1 (FPR1) Gene in Primary Human Macrophages".

1.1.7 Resolution of inflammation

The players of acute inflammation described in this chapter are of pivotal importance to defend our body against pathogen invasion or tissue injury. Acute inflammation is however an unstable state, it either resolves after the noxious agents are eliminated or persists leading to chronic inflammation. Traditionally it was thought that inflammation would terminate as a consequence of the removal of the mediators that started the inflammatory response in first place. This "burn out" of inflammatory stimuli theory implicated a mere passive restoration of homeostasis [95]. However with the time more and more anti-inflammatory agents have been discovered, such as IL-10, steroids, adenosine, and T regulatory cells, challenging the pure

passive resolution theory [8]. Another step forward came from studying exudates from self-resolving inflammatory models using untargeted and targeted lipidomic approaches [5]. It was observed that cell-cell interactions give rise to pro-resolution mediators which limit further infiltration of pro-inflammatory neutrophils and increase the phagocytic ability of macrophages to take up apoptotic cells from the tissue. A key step in this active resolution process is the so called lipid mediator class switching in exudates, which is the major focus of the next chapter.

1.2 Lipid mediators

1.2.1 Lipid Mediators in inflammation

Until three decades ago lipids were thought to have exclusive roles as an energy source and as a main component of the cell membrane. Lipids have actually much more than mere structural and energetic functions, they play important roles as signalling and regulatory molecules in almost every physiological process [96]. Some lipid mediators derived from essential fatty acids act as chemical messengers through specialized GPCRs during the course of inflammation. A fact underlined by the malfunction of lipid mediator signalling in inflammatory diseases, cancer, metabolic syndrome, degenerative diseases and many others (For a review see [96]).

In the next chapters we will take a closer look at lipid mediators derived from two classes of fatty acids involved in inflammation: the arachidonic acid (AA) derived lipid mediators and the omega-3 polyunsaturated fatty acid (PUFA) derived lipid mediators.

1.2.2 The AA derived lipid mediators

AA is an omega-6 fatty acid which can be released from membrane phospholipid stores upon different physiological stimuli, via the activation of phospholipase A2 (PLA2) [97]. AA can then be metabolized to distinct lipid mediators via the cyclooxygenase pathway (COX), the lipoxygenase pathway and to a minor extent the Cytochrome P450 monooxigenase pathway (CYP) [98], as shown in Figure 1.

Pro-inflammatory AA-derived lipid mediators

COX enzymes can convert AA to prostaglandins, prostacyclins and thromboxanes. Prostaglandins can be formed by most cells of our body during the initial phase of inflammation and act on specific GPCRs mainly present on endothelial cells and leukocytes [99]. They induce pain, fever and vasodilatation, typical characteristics of the cardinal signs of inflammation [100]. Notably prostaglandin synthesis is the target of the most commonly used anti-inflammatory drug worldwide, aspirin, which acetylates the COX enzyme preventing the formation of prostaglandins and promoting the formation of LXA4 precursors [101].

The lipoxygenase pathway is instead involved in the synthesis of leukotrienes. In an initial step AA is oxygenated to LTA4 by the 5 lipoxygenase (5LOX), which can be further

converted either to cysteinyl leukotrienes or to LTB4 [102]. Cysteinyl leukotrienes elicit changes in calcium and cAMP concentrations leading to pro-inflammatory actions via the specific GPCRs $CysLT_1$ and $CysLT_2$ [103]. LTB4, one of the most potent neutrophil chemoattractants, binds instead BLT1, inducing NF-κB and chemotaxis [104].

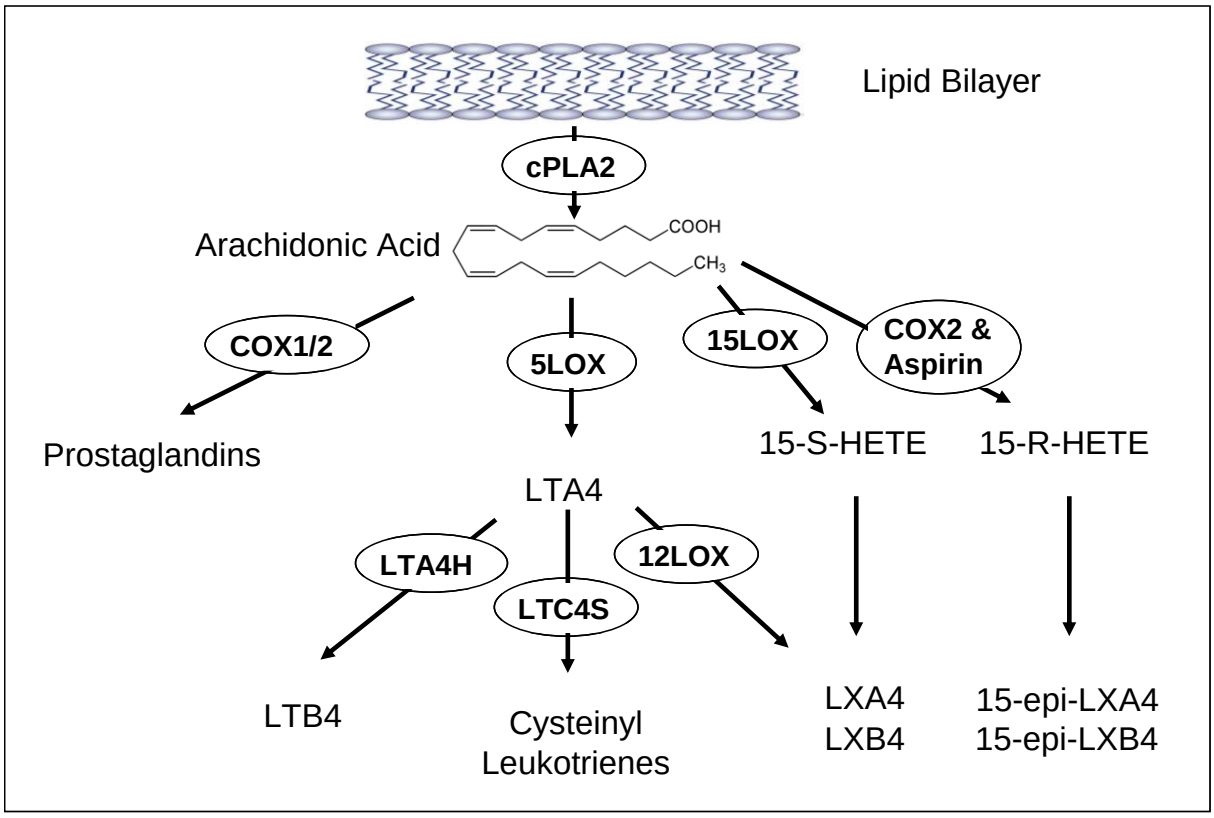

Figure 1: AA derived lipid mediators

Adapted from [105]. Biosynthetic pathways for lipid mediators derived from AA. cPLA2, cytosolic phospholipase A2; COX1/2, cyclooxygenase 1 & 2; 5LOX, 5-lipoxigenase; 15LOX, 15-lipoxigenase; LTA4H, leukotriene A4 hydrolase; LTC4S, leukotriene A4 synthase, 12LOX, 12-lipoxigenase; LTB4, leukotriene B4; 15-S-HETE, 15-S-hydroxy-eicosatetraenoic acid; 15-R-HETE, 15-R-hydroxy-eicosatetraenoic acid; LXA4, lipoxin A4; LXB4, lipoxin B4.

Pro-resolution AA-derived lipid mediators: the lipoxins

Lipoxins have been identified as a novel class of lipid mediators with pro-resolution actions in 1984 [106]. Their biosynthesis is regulated by three major pathways: the 5LOX pathway, the 15LOX pathway and the acetylated COX2 pathway. Oxigenation of AA by 5LOX in neutrophils leads to the intermediate metabolite LTA4, which is released and taken up by platelets to form LXA4 and lipoxin B4 via a further oxygenation by 12LOX [107, 108]. Interestingly, production of lipoxins via this pathway blocks the formation of leukotrienes, leading to an opposite regulation and possibly to a lipid mediator class switching [109]. The

second biosynthetic route involves the direct oxygenation of AA by 15LOX, leading to the generation of 15S-hydroxy-eicosatetraenoic acid (15-S-HETE). Other than being the precursor for the more potent lipoxins, 15-S-HETE has direct anti-inflammatory actions [110, 111]. The last pathway involves aspirin, which acetylates the COX-2 enzyme suppressing prostaglandin synthesis. Acetylated COX-2 produces instead 15-R-HETE, which can be further converted by 5LOX to 15-epi-lipoxins, also known as aspirin-triggered (AT) lipoxins [101].

LXA4 and its aspirin triggered isomer display a wide range of anti-inflammatory and pro-resolution actions. They have been shown to block neutrophil chemotaxis, adhesion and transmigration trough the endothelium [112], to increase monocyte chemotaxis and non-phlogistic phagocytosis of apoptotic neutrophils [113]. Lipoxins also display potent actions *in vivo* and are protective in several animal models of inflammation, reducing neutrophil infiltration and tissue damage and increasing survival (reviewed by Gonzalez-Periz and Claria [114]).

1.2.3 The omega-3 PUFA derived lipid mediators

The n-3 essential fatty acids eicosapentanoic acid (EPA) and docosahexanoic acid (DHA) are of great interest and a large body of literature illustrating their anti-inflammatory effects of already exists (for review see [115]), however the molecular mechanism addressing these anti-inflammatory properties is still object of intense investigation. Neither EPA nor DHA is produced in humans to any great extent, however, they can be supplied in the diet, especially with marine oils [116]. Furthermore both essential fatty acids were found to be substrates for the biosynthesis of potent pro-resolution mediators identified in resolving exudates [117, 118]. EPA is converted to 18-HEPE, the precursor of the resolvins of the E series, via either the acetylation of the COX2 enzyme by aspirin [118] or through the cytochrome P450 pathway [119]. DHA is instead the precursor for protectins [119], the recently identified maresins [120], and resolvins of the D series (Figure 2), whose biosynthetic pathway is discussed in chapter 1.3.1.

The two omega-3 metabolites best characterized so far are RvE1 and RvD1, which control the magnitude and duration of inflammation in animals and function as agonists of resolution during the different stages of inflammation [121]. These two specialized pro-resolution mediators are therefore object of intensive investigations for possible therapeutic approaches in humans.

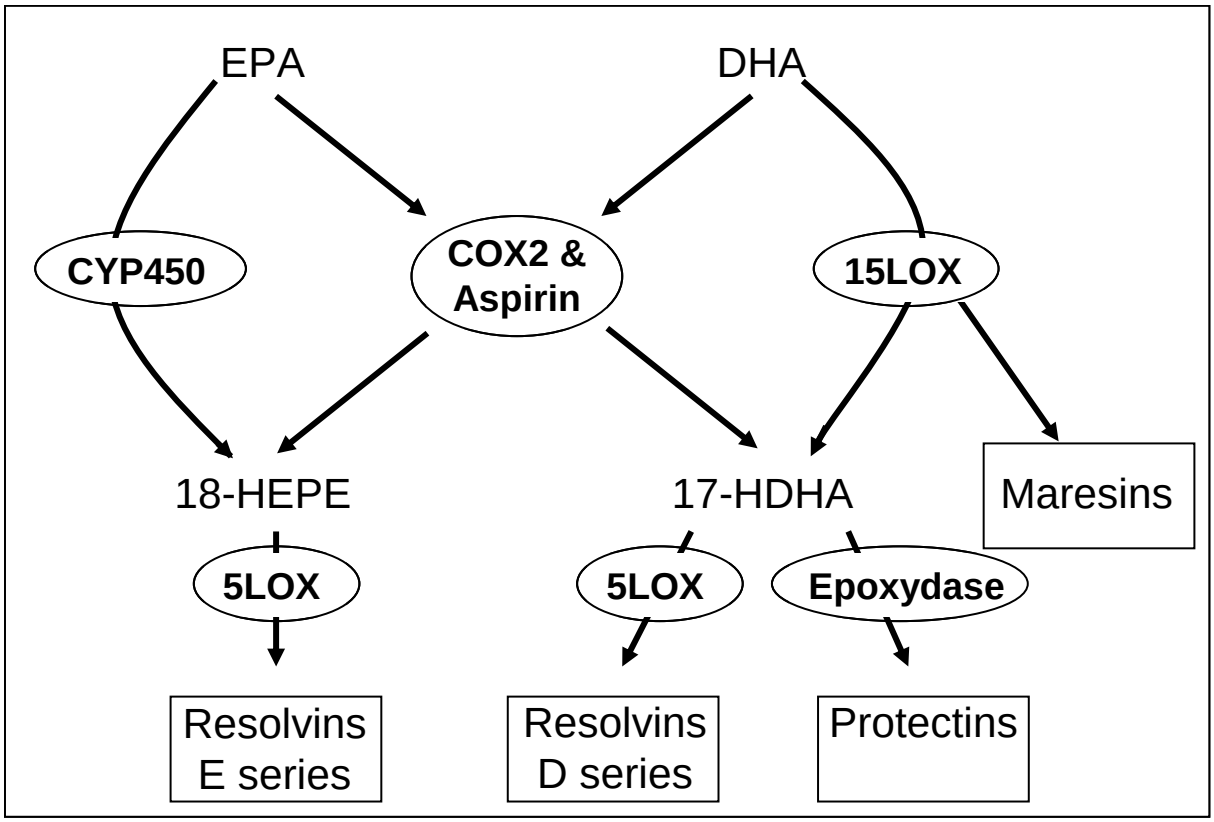

Figure 2: Biosynthesis of resolvins

Adapted from [105]. Biosynthetic pathways of resolvins, protectins and maresins. EPA, eicosapentanoic acid; DHA, docosahexanoic acid; CYP450, cytochrome P450 ;COX2, cyclooxygenase 2; 15LOX, 15-lipoxigenase; 18-HEPE, 18R-hydroxy-eicosapentaenoic acid; 17-HDHA, 17hydroxy-docosahexaenoic acid; 5LOX, 5-lipoxigenase.

1.2.4 Lipid mediator time-line in inflammation

Once inflammation starts, it gets amplified through a loop of pro-inflammatory signals until the insulting agents (infection or injury) are contained. At this time point it is crucial for the body to initiate the resolution of inflammation to avoid establishment of chronic inflammation and to promote the restoration of homeostasis. The lipid mediator class switching is at least in part responsible for controlling this inflammatory process [122]. During the initial phase of inflammation mediators such as prostaglandins are produced which regulate blood-flow and vaso-permeability. However, signalling of PGE_2 and PGD_2 also leads to the transcription of the enzymes required for the biosynthesis of lipoxins and later resolvins [122]. These newly produced mediators promote then the resolution of inflammation by reducing neutrophil infiltration, recruiting non-phlogistic monocytes and favouring the removal of apoptotic neutrophils by macrophages (Figure 3).

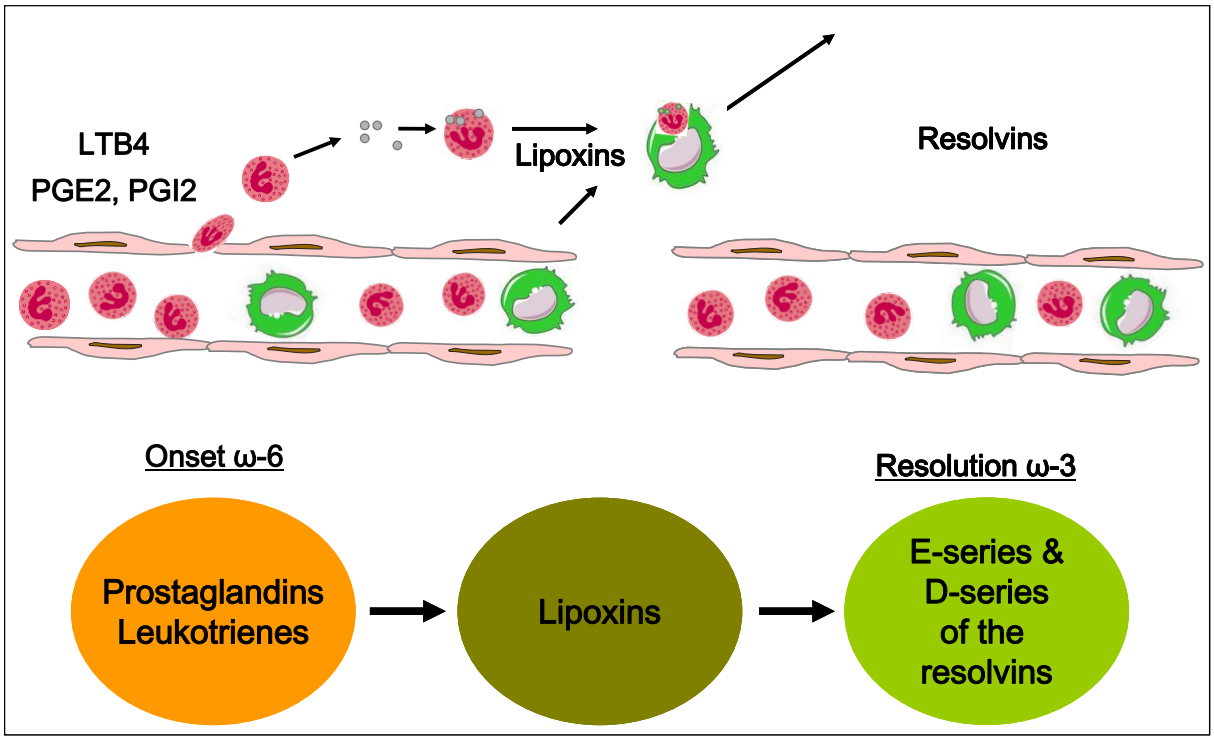

Figure 3: Lipid mediator class switching during inflammation

Adapted from [123]. After tissue injury or pathogen invasion, prostaglandins increase the vasopermeability and LTB4 attracts the pro-inflammatory neutrophils into the affected tissue. Once the noxious agents have been eliminated by neutrophils, a lipid mediator class switch to first lipoxins and then resolvins ensures that no further neutrophils are recruited and that restoration of the homeostasis is promoted via the non phlogistic phagocytosis of apoptotic cells in the tissue.

1.3 Resolvin D1

RvD1 is a DHA derived lipid mediator, which was first isolated during the resolution phase of inflammation from mice treated with aspirin [118]. This potent mediator controls the magnitude and duration of inflammation in several animal disease models and ameliorates several inflammatory pathologies affecting the airways, vasculature, adipose tissue, renal and ocular systems, and regulates physiological processes such as pain, fibrosis and wound healing (for review see [124]). Its role in controlling neutrophil influx, promoting pro-resolution macrophages, and in decreasing pro-inflammatory mediators seems to be pivotal in inflammation in almost all organs.

1.3.1 The biosynthesis of RvD1

The LC-MS/MS lipidomic analysis of resolving exudates from mice given DHA and aspirin identified the new endogenous lipid mediator 17R-HDHA [118]. The biosynthetic pathway leading to the production of this new compound was unveiled using isolated human cells and recombinant enzymes. The COX2 enzyme converts DHA to 13-hydroxy-DHA in hypoxic endothelial cells, however, aspirin acetylated COX2 enzyme converts DHA to 17R-HDHA. Human neutrophils then further oxygenate 17R-HDHA with the 5LOX enzyme to the trihydroxy-containing mediators, which were named aspirin triggered D series resolvins (AT-RvD1). Remarkably, D series resolvins were also identified in murine exudated and isolated human cells not treated with aspirin [117, 118], a biosynthetic pathway which involves the 15LOX enzyme (Figure 4). The stereochemistry of RvD1 was established after its complete organic synthesis [125] and is shown in Figure 4. Several additional members of the D series resolvins arising from similar biosynthetic pathways but with distinct chemical structures and bioactions have been identified. For example RvD2 and RvD5 are protective against bacterial infections and sepsis [126, 127], while RvD3 appears during a unique timeframe of the late resolution phase, suggesting for a specialized role [128].

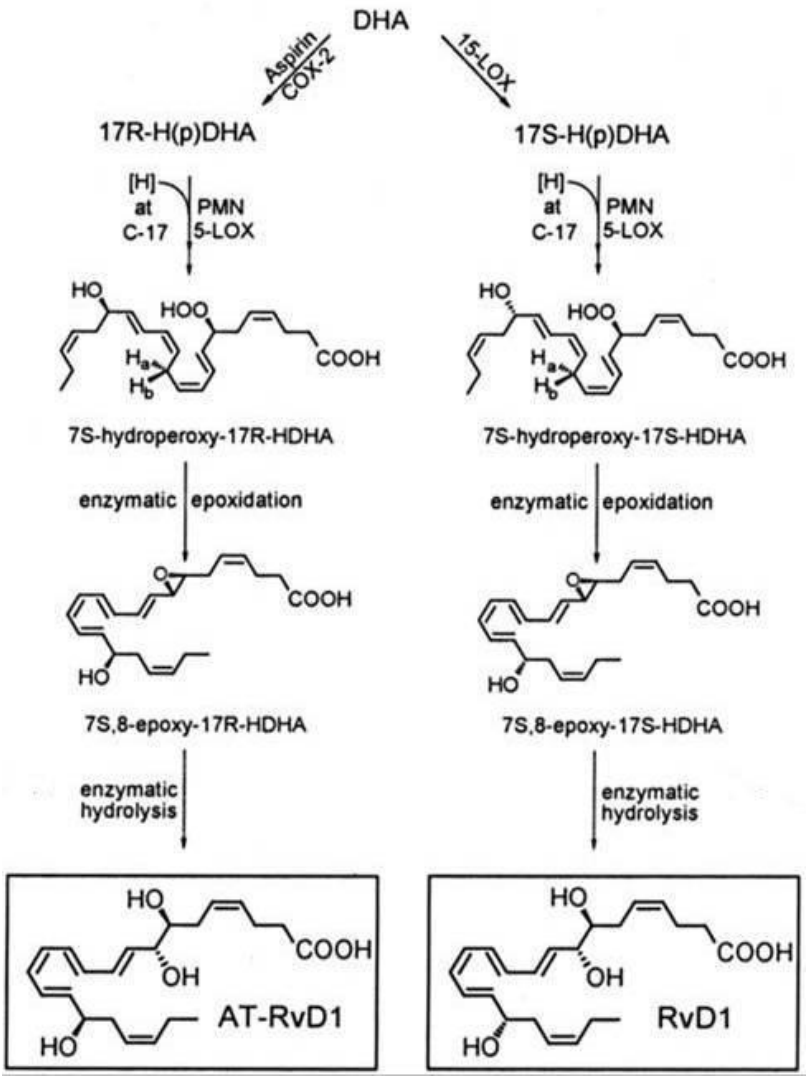

Figure 4: RvD1 and AT-RvD1 biosynthesis [125]

Proposed pathways for RvD1 and AT-RvD1. RvD1 and AT-RvD1 are both generated from DHA as a product of transcellular biosynthesis between leukocytes and endothelial cells. In a first step 15LOX converts DHA to 17*S*-H(p)DHA, whereas aspirin-acetylated cyclooxygenase-2 generates predominately *R*-containing 17*R*-H(p)DHA in endothelial cells. Then the 17-hydro(peroxy) products of DHA can be converted rapidly in neutrophils by 5LOX to 7,8-epoxide-containing intermediates. Enzymatic hydrolysis of each 7,8-epoxy-intermediate follows to form the bioactive RvD1 and AT-RvD1.

1.3.2 RvD1 induces pro-resolution actions on leukocytes in experimental inflammation

RvD1 has a wide spectrum of anti-inflammatory and pro-resolution actions in several animal models of diseases, summarized in Table 1. In order to define RvD1 actions in inflammation and its resolution, resolution indexes which measure changes in inflammatory exudates have been coined. The most important value in this context is the resolution interval, which encompasses the time needed to have a 50% reduction of neutrophils in exudates in respect to the time at which maximum infiltration occurs [7]. The use of these resolution indices allows

to quantify the pro-resolution actions of endogenous mediators or pharmacological mimetics [129-131].

	Animal	Disease Model	Mechanism of action	Ref
RvD1	Mouse	Peritonitis	-Reduces neutrophil infiltration -Reduces prostaglandins and leukotrienes in exudates -Shortens resolution interval	[117, 125, 132-135]
		Dorsal air pouch	-Reduces neutrophil infiltration	[117, 118]
		Corneal Inflammation	-Reduces leukocyte infiltration and hemangiogenesis	[136]
		E. coli / S. aureus infection	-Reduces bacterial titres and hypothermia -Shortens resolution interval -Increases microbial containment -Increases survival	[126]
		Retinopathy	-Protective against neovascularization	[137]
		Acute lung Injury	-Reduces leukocyte infiltration -Reduces cytokines in bronchoalveolar lavage fluid	[138]
		Kidney ischemia-reperfusion	-Reduces kidney damage and protects from loss of function -Blocks TLR activation of macrophages	[139]
		Obesity	-Induces resolution of adipose tissue inflammation -Polarize adipose macrophages to a pro-resolution phenotype -Reduces adipose macrophage cytokines	[140, 141]
		T2 diabetes	-Improves insulin sensitivity -Reduces macrophages accumulation -Promotes healing of diabetic wounds	[142, 143]
		Inflammatory pain	-Reduces spontaneous pain -Reduces mechanical pain and heat hypersensitivity	[144-146]
	Rat	Uveitis	-Reduces leukocyte infiltration -Reduces cytokines	[147]
		Inflammatory pain	-Decreases mechanical allodynia in colitis	[148]
		Post-operative pain	-Reduces tactile pain sensitization -Reduces hyperalgesia	[149]
AT-RvD1	Mouse	Colitis	-Reduces neutrophil infiltration -Reduces disease activity indexes	[150]
		Temporoman-dibular inflammation	-Blocks Freund's adjuvant-induced neutrophil infiltration	[151]
		Pain	-Attenuates pain signals and behaviour	[152]
	Rat	Arthritic pain	-Decreases hyperalgesia -Decreases TNFα and IL-1β	[153]

Table 1: Bioactions of RvD1 in animal disease models

One of the major actions of RvD1 is a reduction in neutrophil recruitment, a fact that is also seen from the shortened resolution intervals in zymosan-induced peritonitis in mice [134]. Several additional studies, including zymosan-induced peritonitis [117, 125, 132, 133, 135], air-pouch inflammation [117, 118], and E. coli or S. aureus infections [126], confirmed the anti-neutrophil actions of RvD1. Importantly, the dampening of neutrophil inflammation by RvD1 does not involve cell toxicity or immunosuppression but rather the fine-tuning of the immune reaction [8]. These in-vivo observations are in line with experiments on isolated human cells. An example is the reduction of directed human neutrophil chemotaxis towards IL-8 [154]. RvD1 regulates in fact actin polymerization in human isolated neutrophils in a FPR2/ALX receptor dependent manner, and dampens neutrophil adhesion to the endothelium trough a reduction of the surface expression of CD11b [58].

The second major target of RvD1 actions are macrophages, cells which not only have a key role in immunity but also in tissue and metabolic homeostasis [155]. In contrast to the anti-inflammatory actions against neutrophil infiltration, RvD1 acts more as a pro-resolution agent on macrophages. At nanomolar concentrations RvD1 is in fact a strong inducer of macrophage phagocytosis of apoptotic leukocytes [58, 156], yeast particles [58] and bacteria [126], therefore promoting the return of tissue homeostasis. Interestingly upon administration of RvD1 to a murine peritonitis model, less macrophages were found in the peritoneum [157], indicating that RvD1 may be able to block not only neutrophil chemotaxis but eventually macrophage chemotaxis also.

1.3.3 RvD1 induces resolution phase macrophages *in vivo*

Another effect of RvD1 is the polarization of macrophages towards a pro-resolution phenotype in mouse. Pro-resolution macrophages have been observed *in vivo* during the resolution phase of inflammation in different mouse models [39, 141, 157, 158]. These pro-resolution macrophages express high levels of CD206 and arginase-1, secrete lower levels of inflammatory cytokines and higher levels of the anti-inflammatory IL-10, all proprieties of M2 macrophages. They display, however, also M1 characteristics, such as high expression of iNOS and COX2 [39]. Transcriptomic analysis of these pro-resolution macrophages also showed high levels of IL-10, COX-2 and CD206, and despite the low secretion, also high levels of mRNAs of inflammatory cytokines [158].

Intraperitoneal injection of RvD1 in the same mouse model further induced the formation of pro-resolution macrophages, with high phagocytic ability, low TNFα and high IL-10 secretion and with lower CD11b expression [157]. In a similar way, RvD1 was also able to repolarise

pro-inflammatory adipose tissue macrophages of obese mice to a pro-resolution phenotype which displayed higher phagocytosis, expressed more arginase 1 and lower pro-inflammatory cytokines [141]. Taken together, the results of these studies indicate that RvD1 is not a mere inducer of phagocytosis of macrophages, but rather a polarizing agent with broader effects.

1.3.4 RvD1 and miRNA-regulated resolution of inflammation

The resolution of inflammation is a highly coordinated process regulated by cytokines, chemokines, lipids and other mediators. An emerging line of investigation indicates that miRNAs are fine tuners of many cellular processes, including the immune response [159, 160]. Indeed, miR146, suppresses the NF-κB pathway in macrophages by inhibiting translation of genes like IκB kinase and tumor necrosis factor receptor-associated factor 6 [132]. Another example is miR-208a, which downregulates PDCD4 (programmed cell death protein 4) [134], a tumor suppressor molecule that acts as a translational repressor of IL-10 [161], and therefore favours the production of this anti-inflammatory cytokine. In contrast, miR-219 directly interferes with the biosynthesis of leukotrienes, binding to the 3'UTR of the 5LOX gene and decreasing its translation [162]. All these miRNAs are upregulated during the resolution phase of inflammation *in vivo* [159] and RvD1 treatment further increase their production (Figure 5) [134]. RvD1 also down-regulates miRNAs, such as miR466I, which was up-regulated in peripheral blood of sepsis patients, and whose increase correlated with non-survival in sepsis [163], even though the mechanism of action is not yet clear. Furthermore, these RvD1 dependent miRNA regulations involve the ALX/FPR2 receptor in mice, since transgenic mice over-expressing the receptor reacted even better to RvD1 with higher miRNA expression and shorter resolution intervals [134], while ALX/FPR2 knockout mouse did not upregulate those miRNAs [132] and showed no reduced leukocyte infiltration in a model of peritoneal inflammation [133].

Taken together these studies indicate that RvD1 regulates miRNAs involved in the fine tuning of inflammation, and that RvD1 mediated miRNA regulation is mediated through specific receptors, which are the topic of chapter 1.5.

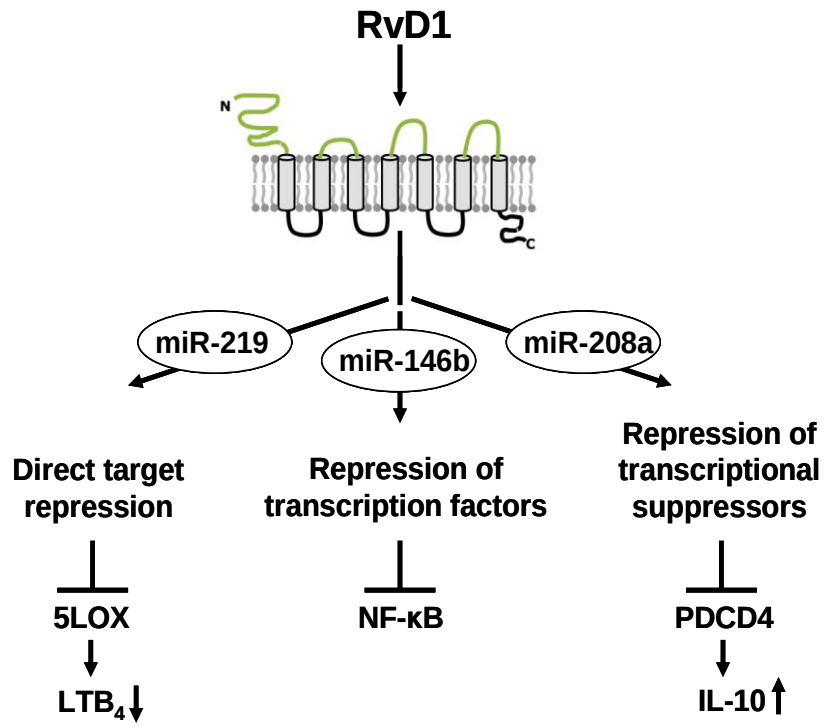

Figure 5: RvD1 induces pro-resolution miRNAs

Adapted from [124]. RvD1 promotes the production of several miRNA in macrophages during the resolution phase of inflammation. These miRNA have different effects which include direct repression of leukotriene production, blocking of the NF-κB transcription factor and reduction of PDCD4, a transcriptional repressor if IL-10. 5LOX, 5-lipoxigenase; LTB4, leukotriene B4; PDCD4, programmed cell death protein 4.

1.4 The potential role of RvD1 in human inflammatory diseases

1.4.1 omega-3 PUFA and RvD1 in chronic inflammation

The beneficial roles of omega-3 PUFA in health were already evident in 1929 [164], and include anti-thrombotic, immuno-regulatory and anti-inflammatory effects [116, 165], which may be able to dampen chronic inflammatory diseases. On this line, il Gruppo Italiano per lo Studio della Sopravvivenza nell' Infarto Miocardico-Prevenzione study could show that the supplementation of omega-3 PUFA reduces the cardiovascular risk for death after a myocardial infarction [166]. This effect may however be due to the beneficial effect of omega-3 with arrhythmias, which acted as a confounder [167]. Additionally these studies have been seen as controversial, since the molecular mechanism of action for the omega-3 PUFA is unknown and because of the high concentrations needed to obtain an effect in vitro [116, 165, 168]. More recent studies showed that resolvins and protectins are generated in humans taking omega-3 PUFA supplements, and in a mouse model over-expressing the omega-3 fatty acid desaturase [115, 116] usually not present in mammals. These studies suggested that the generation of resolvins and protectins may explain some of the beneficial effects of the supplementation with omega-3 PUFA.

RvD1 in non-resolving adipose tissue inflammation

Obesity is a dominant risk factor for the metabolic syndrome and related co-morbidities [169]. When the adipose tissue enlarges because of nutrient excess, the supply of oxygen is not able to match the demand of enlarged adipocytes, triggering a hypoxic status in the tissue which is at least in part responsible for the low-grade chronic inflammation [170]. This adipose inflammation is characterized by a rise in pro-inflammatory adipokines such as leptin and cytokines like TNFα, IL-1β, IL-6 and MCP-1 and by a reduction of the anti-inflammatory and insulin-sensitizing adiponectin [171]. This inflammatory milieu promotes the recruitment of inflammatory cells, especially macrophages, inducing an inflammatory vicious cycle. In addition to the increase in pro-inflammatory adipokines, there is also evidence that the level of pro-resolution mediators is impaired in obese adipose tissue. In the subcutaneous and visceral fat of obese and obese/diabetic mice, RvD1, protectin D1 and 17-HDHA were reduced [172, 173]. This reduction was accompanied by an up-regulation of the 15-PG-dehydrogenase/eicosanoid oxidoreductase which has been shown to convert RvD1 to its inactive ketone state (8-oxo-RvD1 and 17-oxo-RvD1) [140], suggesting an increased turn-

over of RvD1 in inflamed adipose tissue. The increased catabolism of RvD1 promoted interest in using this lipid mediator to try to resolve adipose inflammation. Indeed, in mouse fat explants RvD1 rescued the unresolved inflammatory phenotype by inducing secretion of adiponectin, and by reducing the pro-inflammatory adipokine leptin and the cytokines TNFα, IL-1β, and IL-6 [140]. Additionally RvD1 also skewed the classical M1 macrophages of the mouse adipose stromal vascular fraction toward a pro-resolution M2-like phenotype [141] (Figure 6), suggesting its potential to counter adipose tissue inflammation.

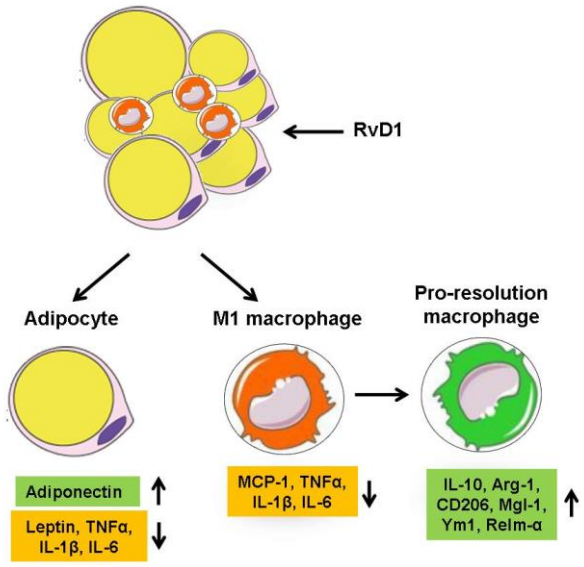

Figure 6: Bioactions of RvD1 on mouse adipose tissue

Adapetd from [174]. In the adipose tissue DHA is endogenously converted to RvD1, evoking pro-resolution actions. In the first place RvD1 polarizes adipose tissue macrophages toward a pro-resolution phenotype, which secretes less pro-inflammatory cytokines and expresses high IL-10 and some typical mouse M2 macrophages markers, such as CD206, Arg-1 and Ym1. In addition to the re-polarization of macrophages, RvD1 also reduces pro-inflammatory cytokine and adipokine production and increases the secretion of adiponectin in adipocytes, an effect which may be mediated by the re-polarized pro-resolution macrophages. Arg-1, Arginase 1; Mgl-1, macrophage galactose N-acetyl-galactosamine (GalNAc) specific lectin; Relm-a; resistin-like molecule a.

RvD1 in hyperglycemia and T2D

The metabolic characteristics of T2D are insulin resistance and hyperglycemia, a common condition related to over-nutrition and obesity [175]. These conditions of nutrient stress lead to an uncontrolled low-grade chronic inflammation, a typical characteristic of T2D and other diseases involving inflammation, such as atherosclerosis [176] and cancer [177].

This correlation between inflammation and T2D is also seen in an obese-diabetic mouse model [143, 178]. In this mouse, macrophages were not able to clear effectively apoptotic thymocytes and IgG-opsonized zymosan. Investigating the mechanism, these authors showed that in the context of over-nutrition PI3K is chronically activated, leading to the impairment of phagosome formation through an increase of the cAMP-PKA axis [179]. RvD1 blocks cAMP accumulation via coupling of FPR2/ALX to Gαi and inhibiting the adenylate cyclase [58, 143, 178], suggesting that it may be able to rescue the reduced phagocytosis. Indeed, in a

recent study, the defect in phagocytosis of diabetic macrophages was reversed by RvD1 in a FPR2/ALX receptor dependent manner [143], leading also to an improvement of insulin sensitivity [142], indicating that RvD1 has the potential to be used in T2D.

The potential vaso-protective actions of RvD1

Vascular inflammation is the underlying cause of atherosclerosis. There is no direct proof of beneficial effects of RvD1 in experimental atherosclerosis yet, however, *in vitro* studies showed that RvD1 blocks proliferation, migration and inflammatory signalling in primary human vascular smooth cells and also reduces monocyte adhesion to those cells [180]. Additionally, isolated endothelial cells stimulated with TNFα, reduced adhesion molecules like VCAM-1 and P-selectin expression when treated before with RvD1 [181]. These RvD1 *in vitro* effects have been further explored recently in an *in vivo* mouse model by Miyahara et al. [180]. The group showed that resolvins of the D series are produced during vascular injury and external administration of resolvins reduced intimal hyperplasia and leukocyte migration into the injured vessel walls. This indicates that RvD1 may be able to ameliorate vascular inflammation, however further studies are needed to proof the vaso-protective role of this lipid mediator and to elucidate its mechanism of action.

DHA and AT-RvD1 in inflammatory bowel disease (IBD)

IBDs are disorders that cause long term impairment of the gastrointestinal structure and function [182] and are characterized by strong leukocyte activation and infiltration into the intestinal tissue, leading to an extensive and unbalanced activation of the whole mucosal immune system [183]. DHA has beneficial effects on experimental IBD, reducing prostaglandins, TNFα, IL-1β and LTB4 [184-186]. Additionally DHA supplementation in humans suffering from distal proctocolitis improved disease activity and histological scores [187] (although the patient numbers in this study were very low). Furthermore, Bento et al. have studied the effects of AT-RvD1 in dextran sulphate sodium and 2,4,6-trinitrobenzene sulfonic acid induced colitis in mice, to clarify the mechanism of action of DHA [150]. AT-RvD1 improved disease activity index, body weight loss, neutrophil infiltration and colon damage, mainly due to a reduction in colonic cytokine levels for TNFα, IL-1β , MIP-2 and CXCL1, as well as due to a reduction in NF-κB and adhesion molecules like VCAM-1, ICAM-1 and LFA-1 [150]. The beneficial effect of DHA in humans suffering from proctocolitis [187], together with the fact that AT-RvD1 ameliorates experimental colitis [150], suggests the potential use of RvD1 and its aspirin triggered isoform in human IBD.

1.4.2 RvD1 and pain

Pain belongs to the cardinal signs of inflammation and is elicited by activation of primary sensory neurons upon stimulation of mediators released from the affected tissue. Among the classical inducers of pain, such as ions, bradykinin and cytokines, the prostaglandins have a major role in the manifestation of this cardinal sign [188, 189]. In order to counteract the effects of prostaglandins, non-steroidal anti-inflammatory drugs which inhibit COX enzymes (for example aspirin and ibuprofen) are commonly used as analgesics [190]. Since RvD1 reduces cytokines and prostaglandin production in mice, its role as an analgesic has been investigated. Low dose RvD1 was as effective as morphine and COX inhibitors to ameliorate pain in different mouse models [145, 146] administered either locally or systemically, without altering basal nociception unlike normal anesthetics. In fact, RvD1 potently inhibited the transient receptor potential cation channel A1 (TRPA1), which contributes to the genesis of pain via both peripheral mechanisms and spinal cord mechanisms [145]. In conclusion, these results show that RvD1 has anti-inflammatory effects (by inhibiting the expression of pro-inflammatory cytokines and prostaglandins) and reduces pain by blocking the TRPA1 channel.

1.5 The receptors of RvD1

Given the potent bioactivities of RvD1 in experimental inflammation, several efforts have been taken to identify possible receptor candidates. To investigate the binding of RvD1 to human leukocytes, synthetic tritiumlabeled [^{3}H]-RvD1 was synthesized. [^{3}H]-RvD1 displayed specific binding to human monocytes and neutrophils with high affinity (K_d = 0.17nM). The homoligand RvD1 as well as LXA4 competed for its binding, displacing 100% [^{3}H]-RvD1, in case of RvD1 and 60% with LXA4, while another FPR2/ALX-ligand, the Ac2-12 peptide derived from Annexin A1 could not [58]. Co-transfection of HEK-293 cells with Gal4-responsive luciferase reporters revealed that RvD1 did not activate the human nuclear receptors PPARα, PPARγ, PPARδ and retinoid χ receptor-α [58], indicating that RvD1 acts through a GPCR. To screen for candidate receptors, phylogenetically related GPCRs associated with inflammation and chemotaxis were co-expressed in HeLa cells with a NF-κB target reporter construct [58]. RvD1 reduced TNFα stimulated NF-κB activation in cells transfected with either the LXA4 receptor FPR2/ALX [191] or the orphan receptor GPR32 [192], but not other GPCRs like BLT1, BLT2, CB1, GPR1, FPR, and ChemR23 [58]. After identifying the receptors for RvD1, a GPCR-β-arrestin-coupled system was used to define the receptor-ligand interactions. In this experimental setup RvD1 activated dose dependently and selectively the FRP2/ALX and GPR32 receptors, with an EC_{50} of 1.2 x 10^{-12} M and 8.8 x 10^{-12} M, respectively [58]. Interestingly, the precursor of RvD1, DHA, did not activate either receptor at similar concentrations [132]. Another study used blocking antibodies to show that low nanomolar RvD1 concentrations preferentially activate GPR32, while concentrations higher than 10nM were needed to activate FPR2/ALX [133].

Functional experiments on isolated human leukocytes further suggest that RvD1 triggers the two receptors. For example over-expression of FPR2/ALX or GPR32 in human macrophages further increased the clearance of apoptotic neutrophils upon RvD1 stimulation, while knock-down of GPR32 resulted in opposite effects [58]. On the same line, the actions of RvD1 on a human neutrophil-endothelial system rely on the 2 receptors [133]. In this experimental setup, the RvD1 inhibited chemotaxis of neutrophils toward endothelial cells was abolished upon blocking the two receptors FPR2/ALX and GPR32 with specific antibodies.

1.5.1 The FPR2/ALX receptor

The FPR2/ALX receptor was first identified during a screening of a cDNA library obtained from the human HL-60 cell line [191]. Initially, due to the high homology with FPR1 (69% sequence identity) it was named formyl-peptide receptor-like 1 (FPRL1) [193], FPRH1 [194] or FPR2 [195]. However since it has low affinity for formylated peptides it was later reclassified in light of its binding to the endogenous ligand LXA4 as FPR2/ALX receptor [24, 196]. Several structurally diverse ligands, including lipid mediators and peptides bind with high affinity to the FPR2/ALX receptor. Among the pro-inflammatory peptides which activate the receptor, serum amyloid A was the first being identified [197]. This peptide induces pro-inflammatory actions which include an increased IL-8 secretion form neutrophils [198] and an increased matrix-metalloproteinase and MCP-1 production in monocytes [199]. FPR2/ALX also binds anti-inflammatory or pro-resolution mediators such as annexin-1 [200] and LXA4 [201], which inhibit neutrophil infiltration [202] and promote monocyte chemotaxis [27, 203, 204]. Additionally, RvD1 also binds with an high affinity to the FPR2/ALX receptor [58, 133], inducing the potent pro-resolution effects in animal models of inflammatory diseases and in isolated human cells, that have been described in the previous chapters. These contrasting outcomes depending on the binding of pro-inflammatory or pro-resolution mediators suggests activation of different signalling pathways which differentially modulate inflammation.

FPR2/ALX is expressed in human cells involved in inflammation, which includes neutrophils [204], monocytes [193], NK cells [205], endothelial cells, epithelial cells [206], but not human macrophages [27].

FPR2/ALX orthologues have been identified in other primates [207], rats [208] and mice [209], and its genetic manipulation in mice provided evidence for its role in regulating the inflammatory response. FPR2/ALX knockout mouse shows an exacerbated inflammatory phenotype with delayed resolution [210], where the pro-resolution action of RvD1, LXA4 and annexin-derived peptide Ac2-26 were nullified [133]. On contrary, a conditional over-expression of the receptor in myeloid cells reduced neutrophil infiltration in zymosan-induces peritonitis [211], confirming the important role of FPR2/ALX in inflammation in mouse.

1.5.2 The GPR32 receptor

The second receptor activated by RvD1 has been cloned more recently from a human genomic DNA screen [192]. In contrast to the FPR2/ALX receptor, an orthologue for GPR32 has been only found in chimpanzee and is not present in mice [58]. This indicates that all the pro-resolution effects of RvD1 seen in mouse models depend on triggering the FPR2/ALX receptor, while in humans both receptors may play a role depending on the cell type. GPR32 was identified in peripheral blood leukocytes, vascular endothelial cells [58] and adipose tissue [140], but cell type specific expression, regulation and signalling have not been investigated so far [140]. Since the FPR2/ALX receptor is absent from primary human macrophages, GPR32 may be the key role player in mediating RvD1 effects in human macrophages.

New LC-MS/MS approaches have confirmed the formation of RvD1 in human tissues, such as breast milk [212], blood leukocytes [213], adipose tissue [214] and brain [215]. The sensitivity of these methods can determine individual human RvD1 levels in various tissues and may be used to determine whether excessive inflammation results from failed resolution because of the lack of RvD1. It is therefore of interest to assess whether the pro-resolution actions of RvD1 which have been seen in animal models can also be translated to humans. The effects triggered by the binding of RvD1 to its high affinity receptor GPR32 can not be studied in mice, due to the lack of the receptor. GPR32 is therefore an interesting candidate for *in vitro* studies, which may highlight its use as a therapeutic target for the RvD1 actions in humans.

2. Results

2.1 Regulation of the formyl peptide receptor 1 (FPR1) gene in primary human macrophages

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2.1.1 Abstract

The formyl peptide receptor 1 (FPR1) is mainly expressed by mammalian phagocytic leukocytes and plays a role in chemotaxis, killing of microorganisms through phagocytosis, and the generation of reactive oxygen species. A large number of ligands have been identified triggering FPR1 including formylated and non-formylated peptides of microbial and endogenous origin. While the expression of FPR1 in neutrophils has been investigated intensively, knowledge on the regulation of FPR1 expression in various polarized macrophages is lacking. In this study we show that primary human neutrophils, monocytes and resting macrophages do express the receptor on their cell surface. Polarization of macrophages with IFNγ, LPS and with the TLR8 ligand 3M-002 further increases FPR1 mRNA levels but does not consistently increase protein expression or chemotaxis towards the FPR1 ligand fMLF. In contrast, polarization of primary human macrophages with IL-4 and IL-13 leading to the alternative activated macrophages, reduces FPR1 cell surface expression and abolishes chemotaxis towards fMLF. These results show that M2 macrophages will not react to triggering of FPR1, limiting the role for FPR1 to chemotaxis and superoxide production of resting and pro-inflammatory M1 macrophages.

2.1.2 Introduction

The formyl peptide receptor 1 (FPR1) belongs to a family of G protein-coupled pattern recognition receptors, which are mainly expressed by mammalian phagocytic leukocytes and are key players in innate immunity and host defense [1,2,3]. In neutrophils, signalling through the FPR1 receptor plays a role in chemotaxis, killing of microorganisms through phagocytosis, and generation of reactive oxygen species [2]. In addition, FPR1 is thought to play a role in sensing of endogenous signals of dysfunctional cells, which should attract leukocytes to the site of inflammation and tissue damage [2].

The first ligands identified for FPR1 were the N-formylated peptides from bacteria, however, later it was found that such N-formylated peptides could also derive endogenously from mitochondria, released as a result of severe cell dysfunction or cell death [1]. However, it is currently not clear whether these mitochondria derived N-formylated peptides are produced in vivo [2]. In addition, a large number of microbial and endogenous peptides of various compositions have been identified as agonists for FPR1. These include formylated and non-formylated peptides of microbial and endogenous origin, like the GP-41 envelope protein of the human immuno-deficiency virus (HIV), annexin-1, and a list of peptides from peptide libraries [2,4].

Several intracellular signalling pathways regulating chemotaxis and superoxide production are triggered by FPR1 in neutrophils [2]. Chemotaxis towards pathogens is mediated by activated Gαi involving the PI3K family of kinases [5,6], while several signalling pathways have been identified for superoxide production necessary for the oxidative burst. Superoxide production was shown to be transduced by PI3K mediated signalling pathways but also Rac [7] and PKC dependent pathways [8] are involved in superoxide production upon FPR1 triggering.

In addition, the receptor is regulated by desensitization upon activation with ligands [9]. After stimulation of the FPR1 receptor, the cellular responses rapidly decline in intensity and the cells become refractory to subsequent stimulations with the same agonist. This mechanism common to G protein-coupled receptors results at least partially from phosphorylation of the agonist-occupied receptor by G protein-coupled receptor kinases, leading to its internalization [10,11].

FPR1 is highly expressed in myeloid cells like neutrophils, monocytes and macrophages [12] and the promoter region of the gene has been characterized identifying a myeloid specific transcription factor necessary for transcription of the gene [13]. While the expression of FPR1 in neutrophils has been investigated intensively, knowledge on regulation of FPR1 expression

in various polarized macrophages is lacking. In this study we investigate the regulation of FPR1 expression and function in primary human macrophages. We show that most polarized macrophages do express FPR1 on their cell surface, while IL-4 and IL-13 polarized M2 macrophages do not have functional FPR1 cell surface expression.

2.1.3 Material and Methods

Material

The recombinant human cytokines IL-1β, IL-3, INFγ, and TNFα as well as Lipopolysaccharide (LPS) and the Toll-Like Receptor (TLR) 3 agonist Poly I:C, were purchased from Sigma-Aldrich (Buchs, Switzerland). IL-4, IL-6, IL-13 were obtained from R&D Systems Europe Ltd. (Abingdon, United Kingdom). The TLR9 ligand, CpG-oligodeoxynucleotides (CpG) was synthesized by Microsynth (Balgach, Switzerland). The TLR7 and TLR8 ligands 3M-001 and 3M-002, respectively, were purchased from 3M Pharmaceuticals (St. Paul, MN, USA). FPR1 antibody (Formyl pepdite receptor 1, anti-human, monoclonal antibody) was purchased from R&D Systems Europe Ltd.

Preparation of human peripheral monocytes and cell culture

White blood cells from healthy blood donors were isolated from commercially available and anonymized buffy coats (Blutspendezentrum Zurich, Schlieren, Switzerland) using Histopaque–1077 gradient (Sigma-Aldrich). Peripheral human monocytes were purified by capturing with anti-CD14 antibody coupled to Macs microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany). Monocytes were seeded with a density of 0.7*106 cells/ml and were cultured for differentiation into macrophages for 7 days at 37° and 5% CO2 in RPMI-1640 (Sigma-Aldrich) supplemented with 5% Fetal Calf Serum (Bioconcepts, Allschwil, Switzerland), 5% Human AB Serum (Sigma-Aldrich) and 1% Penicillin/Streptomycin (Invitrogen, Zug, Switzerland). For the stimulation experiments with the set of cytokines, the cells were washed once with PBS (Bioconcept) and incubated in RPMI-1640 (Sigma-Aldrich) supplemented with 5% Human AB Serum (Sigma-Aldrich), 1% Penicillin/Streptomycin (Invitrogen) and the indicated cytokines for different periods.

Quantitative Real-Time PCR (qPCR)

Total amount of RNA was extracted using RNeasy Mini Kit (Qiagen AG, Hombrechtikon, Switzerland) and the reverse transcription reaction was performed with 0.5µg RNA in a 20µl reaction using random primer (Invitrogen) with the Superscript III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. The qPCR reaction was done on a LightCycler 480 system (Roche Diagnostics, Rotkreuz, Switzerland) utilizing a hot-start SYBR green method with the following parameters: preheating for 10min at 95°C, followed by 45 cycles of denaturation for 5sec at 95°C, annealing for 10sec at 60°C and extension for 6sec at 72°C. The quantitative PCR included 50ng cDNA, 0.5µM forward and 0.5µM reverse

primer and 5x SYBR green master mix (Roche Diagnostics). The primers were designed using the OLIGO 6.0 software (Molecular Biology Insights, Inc., Cascade, USA) (sequences are listed in supplementary table S1).

FACS analysis

FACS analysis was perfomed using a monoclonal PE-labeled anti-human FPR1/FPR Ab and an IgG2a isotype control (both R&D Systems, Minneapolis, MN, USA). As a positive control for the stimulations of the cells, CD206 and CD80 Ab were used (BD Biosciences, San Jose, CA, USA). Briefly, cells were resuspended in PBS containing 2.5% FCS and incubated in the dark for 30 min at 4°C before analysis on a FACS Calibur Analyzer (BD Biosciences, San Jose, CA, USA).

Chemotaxis assay

A total of 10^5 macrophages were placed on a 96-well membrane (5.7-mm diameter, 5-mm pore size; ChemoTX from Neuro Probe, Gaithersburg, MD) in RPMI 1640 containing 0.1% BSA (Sigma-Aldrich). The cells were allowed to migrate toward the chemotactic factors at the indicated concentrations for 60min. Migrated cells were fixed (2% paraformaldehyde, VWR International AG, Dietikon, Switzerland) and stained with DAPI (Sigma-Aldrich), and migration was quantified as the total pixel count of DAPI-stained nuclei under the fluorescence microscope (one photo per membrane and two replicate wells per experiment). Migration indices were calculated over control values. The Formyl-Methionyl-Leucyl-Phenylalanine peptide (fLMF) (Sigma Aldrich) was used as a positive control.

Characterization of Macrophages

To confirm the polarization of the resting macrophages into the M1 (INFγ, LPS) and M2 (IL-4, IL 13) subtypes following stimulation, we measured the expression of different markers characteristic for the two subpopulations see supplementary figure 1 and [14]. INFγ and LPS stimulation increased the mRNA expression of the pro-inflammatory cytokines TNF-α and/or IL-1β and the cell surface expression of CD80. IL-4 and IL-13 stimulation increased the mRNA expression of the anti-inflammatory cytokine IL-10, reduced the mRNA of the pro-inflammatory IL-1β and TNF-α, and increased the surface marker CD206.

Statistical analysis

Statistical analysis was performed with Microsoft Office Excel 2003 (Microsoft Corporation, Redmond, CA, USA). The level of FPR1 mRNA, protein and chemotaxis were compared using a two-sided t-test and a two-sided p value of <0.05 was considered significant.

2.1.4 Results

Primary neutrophils, monocytes and resting macrophages express high levels of FPR1 on the cell surface (figure 1). To investigate the regulation of the FPR1 gene in leukocytes, primary human macrophages were stimulated with different pro- and anti-inflammatory cytokines or TLR agonists for 24 hours and the relative mRNA expression was measured by qPCR. Compared to the mRNA expression of resting macrophages, stimulation with IL-4 and IL-13 reduced FPR1 mRNA expression, while stimulation with IFNγ, LPS and with the TLR8 ligand 3M-002 further increased the FPR1 mRNA in macrophages (figure 2A). No regulation of FPR1 mRNA levels was seen with IL-3, IL-1β, IL-6, TNFα, the TLR9 ligand CpG, the TLR3 ligand Poly I:C, and the TLR7 ligand 3M-001.

To corroborate the downregulation of the FPR1 mRNA following IL-4 and IL-13 stimulation, we measured the mRNA levels in a time course experiment for 72 hours (figure 2B). Both cytokines reduced the FPR1 mRNA already after stimulation for 24 hours to less than 50% and after 48 hours to less than 20% of the level of resting macrophages (figure 2B).

To investigate whether the regulation on the mRNA level translated into differential cell surface expression of FPR1, we analyzed the individually stimulated human macrophages for FPR1 cell surface expression by FACS analysis (figure 3A). Similar to the results obtained on the mRNA level, we observed a decrease in FPR1 cell surface expression following stimulation of macrophages with IL-4 and IL-13 and an increase following IFNγ stimulation. However, no increase in FPR1 cell surface expression was observed following LPS and TLR8 (3M-002) stimulation, although both of these stimulations led to increased FPR1 mRNA levels. In addition we observed no increase in intracellular FPR1 staining following stimulation of macrophages with these stimuli (data not shown). In contrast, in a time-course experiment, IL-4 and IL-13 both led to sustained reduction in cell surface expression of FPR1 for up to 72 hours following stimulation (figure 3B).

To show that the reduced cell surface expression of FPR1 in IL-4 and IL-13 stimulated human macrophages has functional relevance, we investigated chemotaxis of macrophages towards the FPR1 ligand fLMF (figure 4). As expected from the cell surface expression of FPR1, resting macrophages migrated towards the chemotactic stimulus. In contrast, macrophages stimulated by IL-4 and IL-13, which have reduced mRNA and protein expression of FPR1, showed no chemotaxis towards fLMF. This indicates that IL-4 and IL-13 stimulated M2 macrophages will not respond to FPR1 ligands unlike resting macrophages or pro-inflammatory M1 macrophages stimulated with IFNγ or LPS.

2.1.5 Discussion

We studied the regulation of FPR1 receptor expression in primary human macrophages and observed that neutrophils, monocytes and resting macrophages express the receptor on their cell surface. Polarization of macrophages towards a pro-inflammatory M1 macrophage further increased FPR1 mRNA levels but did not consistently increase protein expression or chemotaxis towards the FPR1 ligand fMLF. In contrast, polarization of primary human macrophages with IL-4 and IL-13 leading to the alternatively activated M2 macrophages, reduces FPR1 cell surface expression and abolishes chemotaxis towards fMLF. These results show that M2 macrophages will not react to triggering of FPR1, limiting the role for FPR1 to chemotaxis and superoxide production in resting and pro-inflammatory M1 macrophages.

Stimulation of FPR1 by TLR4 agonists has previously been observed in mouse macrophages and the mechanism involved in this increase has been investigated [15,16]. These data demonstrated that FPR1 mRNA levels are dramatically elevated in both macrophages and neutrophils following exposure to LPS and that this resulted both from elevated transcription and from stabilization of the FPR1 mRNA [16]. In this line, higher binding for the fLMF peptide has been observed in leukocytes from patients suffering from Crohn's disease or emphysema indicating that FPR1 expression on leukocytes may serve as a marker for systemic inflammation [17,18]. Our data in human primary macrophages corroborate these findings and extend the number of stimuli leading to FPR1 upregulation by additionally identifying IFNγ and triggering of TLR8 as effective stimuli. IFNγ has been shown to induce effects resulting in heightened immune surveillance and immune system function during infection and to promote M1 macrophage polarization and activation [19]. In contrast, TLR8 signalling is thought to be rather specific for triggering immune responses via recognition of viral RNA in infected cells. TLR8 is one of pattern recognition receptors which is triggered by viral single stranded RNA and which was shown to stimulate macrophages to secrete IFNα and pro-inflammatory, as well as regulatory cytokines [20]. Since both of these stimuli increase FPR1 expression it is tempting to speculate that FPR1 may have a role in host responses to viral infections in addition to its proposed role in fighting bacterial infection. In this line the GP-41 envelope protein of the human immuno-deficiency virus (HIV) [21] and a secreted peptide from Herpes simplex virus-2 (HSV) [22] were shown to specifically activate the FPR1 receptor, and hence to modulate innate immunity during viral infection. However, whether FPR1 has a role in immune surveillance or whether HIV-1 and HSV-2 have evolved to desensitize the effector functions of FPR1, is not known [23].

While the pro-inflammatory stimulation of macrophages raises or at least conserves FPR1 cell surface expression, stimulation of macrophages with IL-4 and IL-13 abolishes FPR1 receptor expression and functionality in polarized M2 macrophages. Similar results were obtained on the mRNA level when mouse peritoneal macrophages were stimulated with IL-4 or IL-13 [24]. In these mouse macrophages, IL-4 and IL-13 suppressed the expression of FPR1 mRNA by a Stat6 dependent mechanism, which eliminated primary transcripts prior to maturation and depended on the constitutive instability of pre-existing FPR1 mRNA [24]. Our results expand these findings showing that also cell surface expression of the receptor and chemotaxis towards fMLF are abolished in M2 macrophages. Such a downregulation by the Th(2) cytokines is in line with the presumed role of FPR1 as a pro-inflammatory receptor supporting chemotaxis and superoxide production in leukocytes. While microbial peptides act as chemoattractants to recruit neutrophils to the site of challenge, where they phagocytose invading microorganisms to protect the host, the acute inflammation has a programmed fate to resolve inflammation upon clearance of the microorganisms to protect the host against unnecessary tissue damage [25]. Resolution of inflammation is part of this program to promote the homeostatic restoration of normal tissue structure and function, and IL-4 is a prototypic mediator of such activity which is known to promote the development of a Th(2) response [26,27]. Downregulation of FPR1 in M2 macrophages indicates that FPR1 may not be necessary for the non-phlogistic alternative macrophage clearing the inflamed tissue from apoptotic cells and microorganisms [28,29].

In summary, we show that primary human neutrophils, monocytes and most polarized macrophages do express FPR1 on their cell surface, while IL-4 and IL-13 polarized M2 macrophages do not express functional FPR1 on their cell surface. These data argue for a role of FPR1 in resting and classically activated macrophages to support chemotaxis and superoxide production, while non-phlogistic alternative macrophages cannot use it.

2.1.6 References

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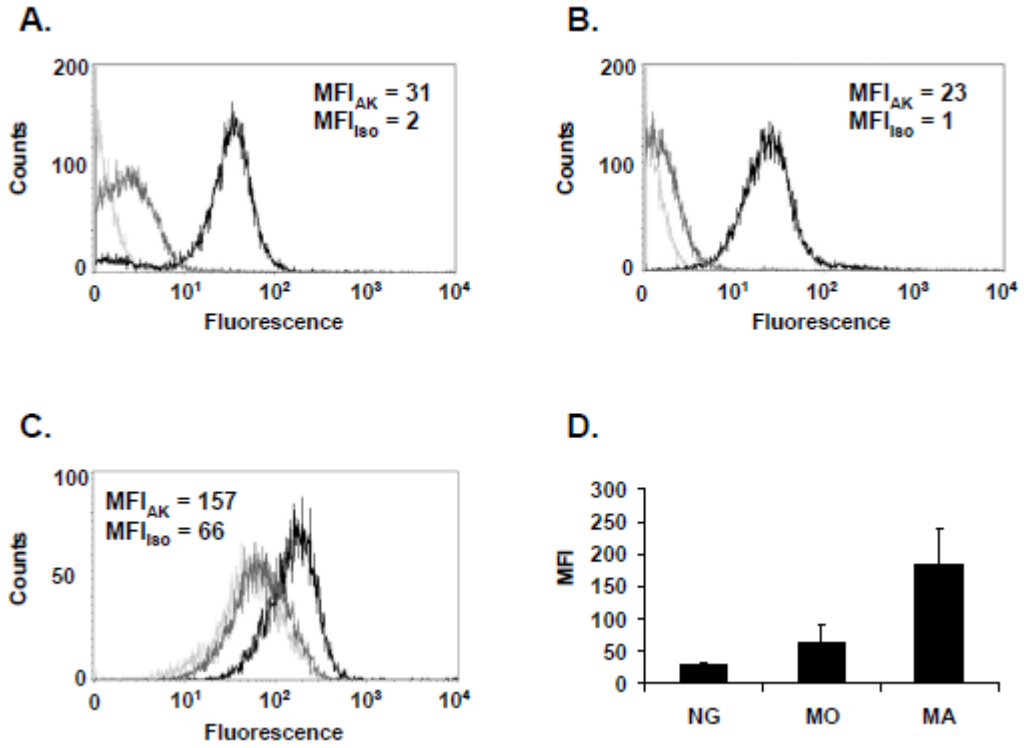
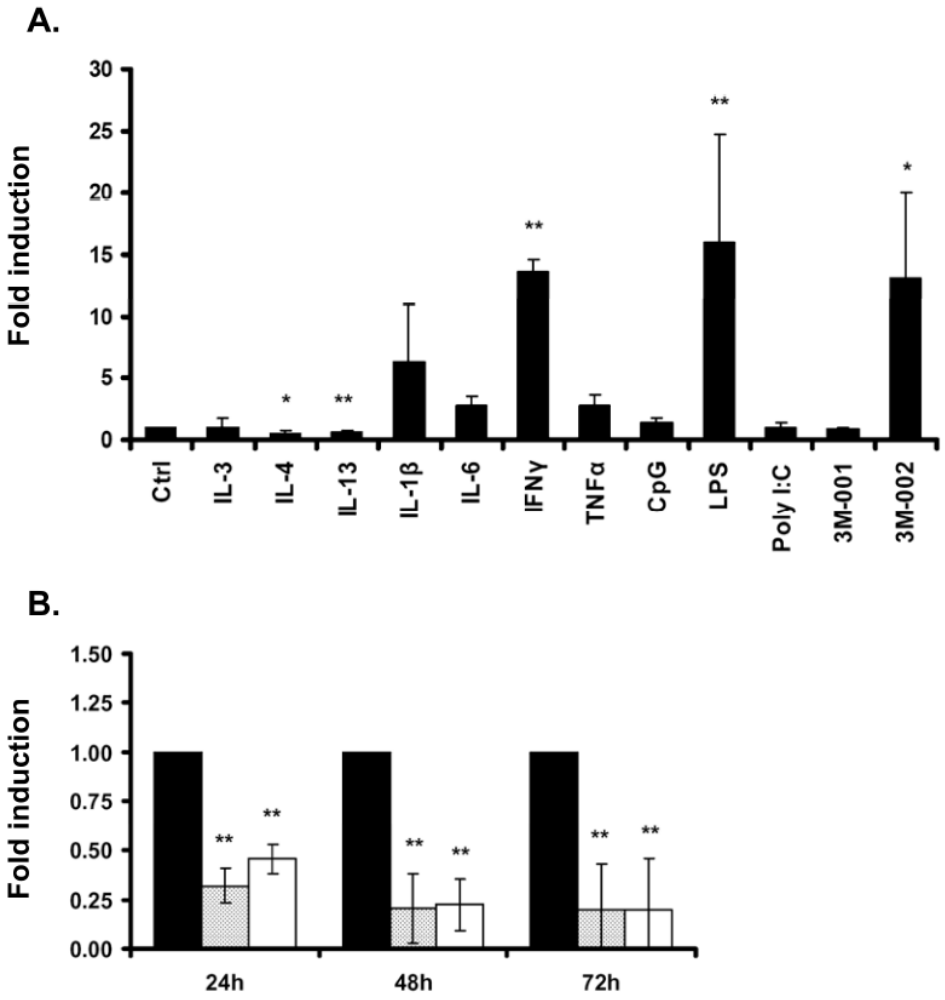
2.1.7 Figures

Figure 1: FPR1 cell surface expression on human neutrophils, monocytes, and macrophages.



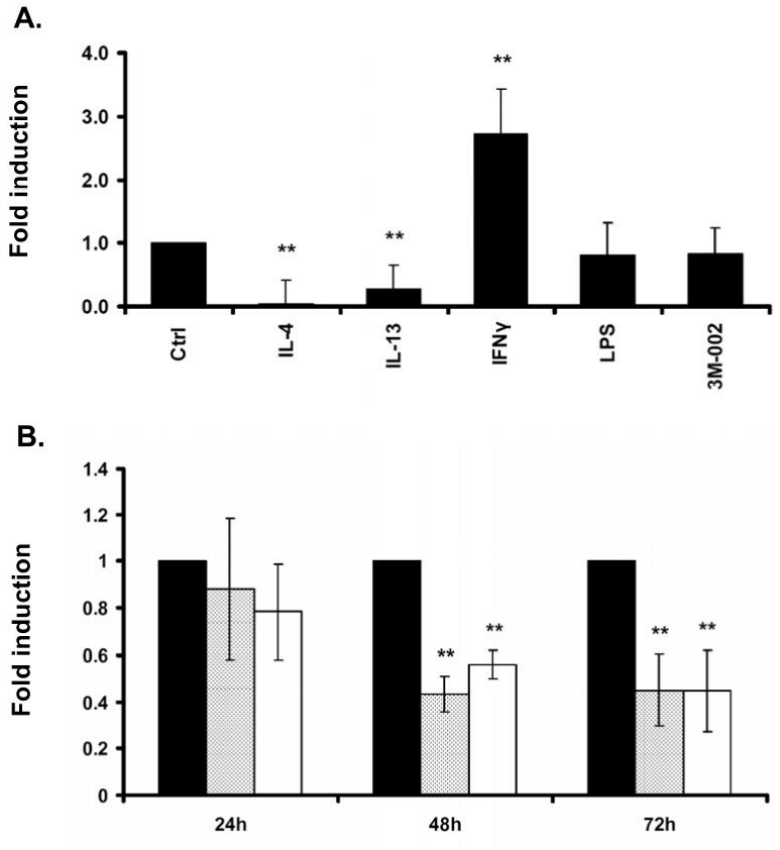
FACS analysis was performed to investigate cell surface expression of FPR1. A) Neutrophils. B) Monocytes. C) 9 day old macrophages. Autofluorescence of the cells is shown in light grey, the isotype control in grey and cells labeled with FPR1 Ab in black. D) Quantitative representation of the FPR1 mean fluorescence intensity (antibody MFI minus isotype MFI) NG: neutrophils (n=3). MO: monocytes (n= 5). MA: 9 day old macrophages (n= 9).

Figure 2: Regulation of FPR1 mRNA expression in human macrophages.



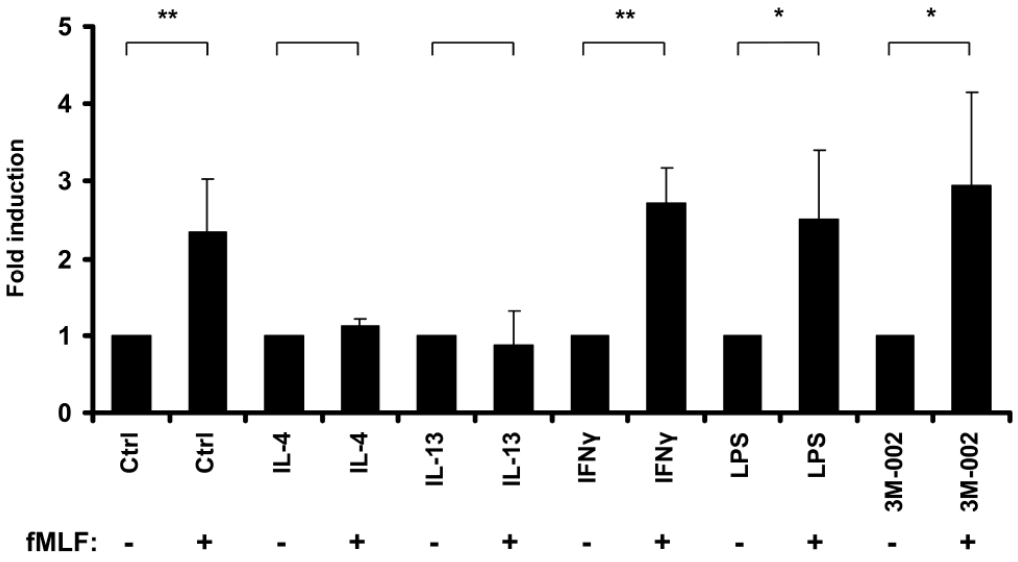
A) Relative mRNA expression of FPR1 after stimulation of human macrophages with different stimuli for 24 hours. IL-3 (20ng/ml), IL-4 (10ng/ml), IL-13 (10ng/ml), IL-1β (5ng/ml), IL-6 (10ng/ml), INFγ (50ng/ml), TNFα (1ng/ml), CpG (100ng/ml), LPS (100ng/ml), Poly I:C (1ng/ml), 3M-001 (3μM), 3M-002 (3μM). B) Time-course experiment of FPR1 mRNA expression in macrophages stimulated with IL-4 (10ng/ml) (dotted) and IL-13 (10ng/ml) (white) for 24, 48 and 72 hours. The values are normalized for GAPDH mRNA expression and are presented relative to non-stimulated control macrophages (black). Bars display the mean and the standard deviation (± SD) of three independent experiments. *p<0.05, **p<0.01.

Figure 3: Regulation of FPR1 cell surface expression in human macrophages.



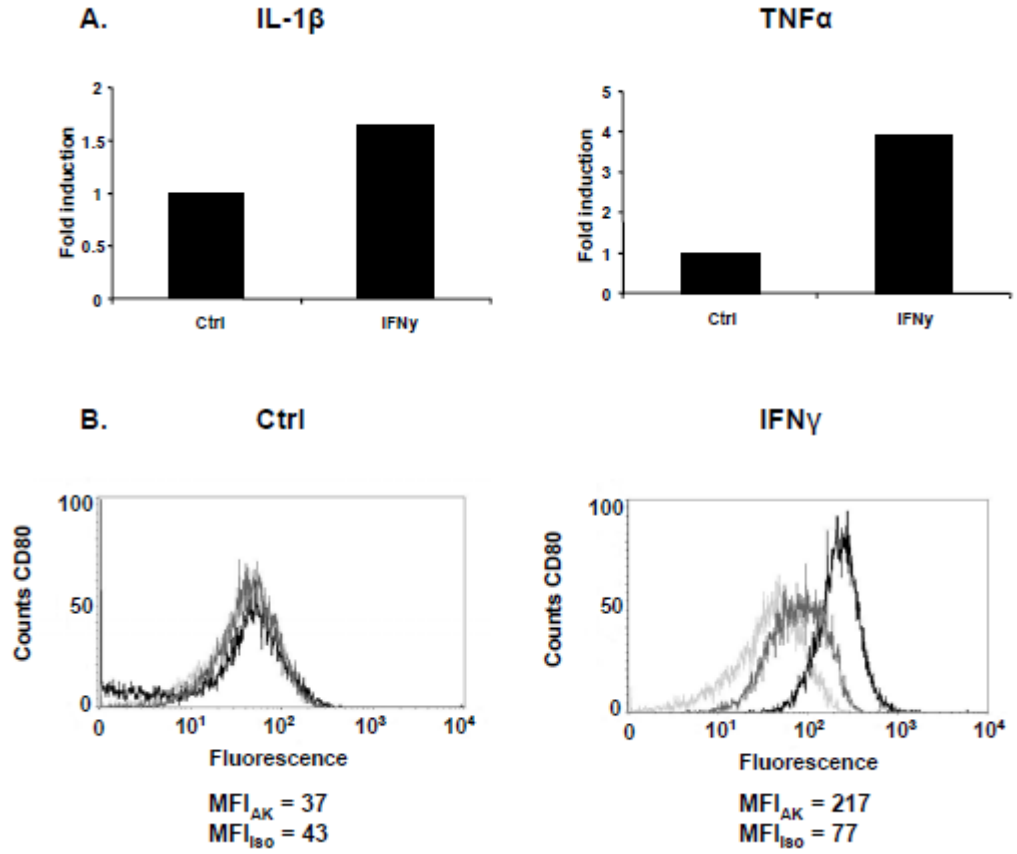
FACS analysis was performed to investigate cell surface expression of FPR1 upon treatment with the indicated stimuli. A) FPR1 protein expression after stimulation of human macrophages for 48 hours with stimuli which were shown to regulate FPR1 mRNA expression (IL-4 (10ng/ml), IL-13 (10ng/ml), INFγ (50ng/ml), LPS (100ng/ml), 3M-002 (3μM)). B) Time-course experiment of FPR1 protein expression in macrophage stimulated with IL-4 (10ng/ml) (dotted) and IL-13 (10ng/ml) (white) for 24, 48 and 72 hours. Values are presented relative to unstimulated macrophages. Bars display the mean and the standard deviation (± SD) of three independent experiments. *$p<0.05$, **$p<0.01$.

Figure 4: fLMF chemotaxis assays with human macrophages.



Control (Ctrl) and IL-4 (10ng/ml), IL-13 (10ng/ml), IFNγ (50ng/ml), LPS (100ng/ml), and 3M-002 (3μM) stimulated macrophages were allowed to migrate towards the chemotactic factor fLMF (+). Medium without chemotactic factor (-) was used as control for basal migration in each experiment. Graphs show the mean migration index compared to each individual control (n=3) and error bars display the standard deviation (± SD). *$p<0.05$; **$p<0.01$.

Supplementary Figure 1



Measurement of the different M1 markers after stimulation of human macrophages with IFNγ (50ng/ml)) for 48 hrs (surface marker CD80) or 24 hrs (relative expression of IL-1β and TNF-α). A: relative mRNA expression of IL-1β and TNF-α normalized for GAPDH as fold induction of non-stimulated cells. B: FACS analysis of CD80 expression on non-stimulated cells (left panel) and on stimulated cells (right panel). Autofluorescence of the cells is shown in light grey, the isotype control in grey and cells labeled with CD80 antibody in black. MFl: Median of fluorescence

2.2 Resolvin D1 polarizes primary human macrophages towards a pro-resolution phenotype through GPR32*

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Key words: GPR32, resolution of inflammation, resolvins, macrophage polarization

Running title: Resolvin D1 triggers GPR32 in human macrophages

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2.2.1 Abstract

Resolvin D1 (RvD1) was shown to be a potent anti-inflammatory and pro-resolution lipid mediator in several animal models of inflammation but its mechanism of action in humans is not clear. We show that the RvD1 receptor GPR32 is present on resting, pro-inflammatory M1 and alternatively activated primary human M2 macrophages, while TGFβ and IL-6 reduce its membrane expression. Accordingly, stimulation of resting primary human macrophages with 10nM RvD1 for 48h maximally reduced the secretion of the pro-inflammatory cytokines IL-1β and IL-8, abolished chemotaxis to several chemoattractants like chemerin, fMLF, and MCP-1, and doubled the phagocytic activity of these macrophages towards microbial particles. In contrast, these functional changes were not accompanied by surface expression of markers specific for alternatively activated M2 macrophages. Similar pro-resolution effects of RvD1 were observed when pro-inflammatory M1 macrophages were treated with RvD1. In addition, we show that these RvD1 mediated effects are GPR32 dependent because reduction of GPR32 expression by siRNA, TGFβ and IL-6 treatment ablated these pro-resolution effects in primary human macrophages. Taken together our results indicate that in humans RvD1 triggers GPR32 to polarize and re-polarize macrophages towards a pro-resolution phenotype, supporting the role of this mediator in the resolution of inflammation in humans.

2.2.2 Introduction

Macrophages are master regulators of inflammation and are key players in the inflammatory response (1). They are the most plastic cell type of the immune system and have an essential role from the initiation to the resolution of inflammation and restoration of homeostasis (2). Upon stimulation macrophages undergo a variety of different activation states, from the pro-inflammatory M1 to the alternatively activated M2, which play different roles in health and disease (3). The Th1 cytokine IFNγ and TLR-4 ligands induce the pro-inflammatory M1 phenotype, which expresses high TNFα and IL-1β levels and produces reactive oxygen and nitrogen species (4). On the other hand, the Th2 cytokines IL-4 and IL-13 lead to the development of M2 macrophages, characterized by high secretion of IL-10 and high expression of CD206 (mannose receptor) (5). The M1 macrophages are known to play an important role in the initiation and progression of inflammation, while the M2 polarization state is associated to the Th2 response against parasite infections, wound healing, tissue repair and restoration of homeostasis (4). M1 and M2 are only the extremes of a continuum of activation states and intermediate phenotypes in specific stages of inflammation such as the resolution phase have been identified (6). In addition, it has been shown that macrophages can to some extend switch between activation states (7, 8).

The lipid mediator 7S,8R,17S-trihydroxy-4Z,9E,11E,13Z,15E,19Z-docosahexaenoic acid named resolvin D1 (RvD1) is produced in resolving exudates from the ω-3 docosahexaenoic acid (DHA) (9). RvD1 is known to block pro-inflammatory neutrophil migration (10) by regulating actin polymerization (11), to reduce TNFα mediated inflammation in macrophages (9), and to enhance phagocytosis of apoptotic cells by macrophages (12). These pro-resolution properties on the cellular level were corroborated in several mouse models of inflammation. RvD1 was shown to reduce inflammatory pain (13, 14), to be protective for airway inflammation (15), to reduce second organ injury induced by ischemia-reperfusion (10), while it enhanced wound healing and suppressed colitis in mouse models of inflammatory diseases (16). RvD1 also lowered adipose tissue inflammation and ameliorated insulin sensitivity in mouse models of obesity by modifying cytokine and adipokine production in adipose tissue, which led to a reduction in macrophage number and in the inflammation state of the fat tissue (17, 18).

The receptors for RvD1 have recently been identified as the orphan receptor GPR32 and the FPR2/ALX receptor (19). While RvD1 triggers both receptors, 4-times lower concentrations are necessary for triggering GPR32 than for triggering the FPR2/ALX receptor (20). The

orphan receptor GPR32 is expressed in human leukocytes and in adipose tissue but cell type specific expression and regulation has not been investigated so far (17). Interestingly, GPR32 has no mouse orthologue, suggesting that the effects seen in mice are depending on FPR2/ALX. In contrast, we have previously shown that the FPR2/ALX receptor is expressed on primary human monocytes but not on macrophages because of alternative promoter usage in macrophages and reduced translation of this mRNA (21), suggesting that GPR32 may be the key player in mediating RvD1 effects in human macrophages.

To better understand the mechanism of RvD1 mediated pro-resolution effects in human inflammation, we analyzed the expression and function of GPR32 in differently polarized primary human macrophages and the effects of RvD1 on macrophage polarization and re-polarization. We show that GPR32 is expressed on the surface of resting, M1 and M2 macrophages and that GPR32 is the receptor triggered by RvD1 in primary human macrophages. We further show that triggering GPR32 induces a pro-resolution macrophage phenotype with reduced secretion of pro-inflammatory cytokines, low chemotaxis and increased phagocytosis of microbial particles.

2.2.3 Material and Methods

Materials

The TLR4 ligand LPS and the TLR3 ligand poly I:C were purchased from Sigma Aldrich (St. Louis, MO, USA). The recombinant human cytokines IL-4, IL-6, IL-13, IL-1β, TNFα, TGFβ, and IFNγ were purchased from R&D Systems, Inc. (Minneapolis, USA). The TLR9 ligand, CpG, was synthesized by Microsynth (Balgach, Switzerland). The TLR7 and 8 synthetic ligands 3M001 and 3M002 were purchased from 3M Pharmaceuticals (St. Paul, Minnesota, USA). The formylated peptide fMLF and MCP-1 were purchased from Sigma Aldrich. Chemerin (149-157) was purchased from AnaSpec (Fremont, USA). GPR32 rabbit anti-human antibody (GPR32 Ab) and its isotype IgG were purchased from GeneTex (Irvine, USA), the secondary AlexaFluor488 anti-rabbit antibody from Life Technologies (Carlsbad, California, USA). PE-labeled anti-human CD80 Ab, allophycocyanin-labeled anti-human CD206 Ab and the IgG1K isotype control were purchased from R&D Systems. RvD1 (7S,8R,17S-trihydroxy-4Z,9E,11E,13Z,15E,19Z-docosahexaenoic acid) produced by stereospecific total synthesis (22) was purchased from Cayman Chemicals (Ann Arbor, MI, USA) and was verified by LC-MS/MS analysis in the laboratory (23).

Primary cell purification and cell culture

White blood cells from healthy volunteers were isolated as described previously (24) from buffy coat (Blutspendezentrum, Zurich, Switzerland) using Histopaque-1077 (Sigma-Aldrich) gradient centrifugation. Briefly, monocytes were purified by capture with anti-CD14 Abs coupled to MACS Microbeads (MiltenyiBiotec, Bergisch Gladbach, Germany) and let differentiate into macrophages for 7d at 37°C and 5% CO2 in RPMI 1640 (Sigma-Aldrich) supplemented with 5% Fetal Calf Serum (Sigma-Aldrich), 5% human AB serum (Sigma-Aldrich) and 1% penicillin/streptomycin (Sigma-Aldrich).

RNA isolation and RT reaction for real-time PCR (Q-PCR)

Total RNA from primary monocytes and macrophages was isolated using the RNeasy Mini kit from Qiagen AG (Hilden, Germany). cDNA was prepared by RT reaction from 1µg total RNA using the SuperScript III reverse transcriptase with random primers according to the manufacturer's instructions (Life Technologies).

Relative quantification of GPR32 expression levels

Each Q-PCR was performed in a total volume of 20µl on a LightCycler 480 II System (Roche Diagnostics, Rotkreuz, Switzerland). GPR32 mRNA quantification included 100ng cDNA, 4 mM MgCl2, 0.5 mM upper primer (5'-TGTGGTTATCCTGTCTGCGTC-3'), 0.5 mM lower primer (5'-GGCCAATGGTCCCTATAATGT-3') and 1x SYBR Green enzyme mix (Roche Diagnostics). The reactions were performed under following conditions: preheating 10min at 95°C followed by 45 cycles of denaturation 5s at 95°C, annealing 10s at 60°C and extension 6s at 72°C. Relative gene expression was normalized to GAPDH (forward primer 5'-CCCATGTTCGTCATGGGTGT-3', reverse primer 5'-TGGTCATGAGTCCTTCCACGATA-3'). Data was analyzed with the Light Cycler 480 software (Roche Diagnostics).

FACS analysis

Briefly, cells were resuspended in PBS containing 2.5% FCS, and Ab or isotype control were added and incubated in the dark for 30min at 4°C. For GPR32 probes and the respective isotype controls, the cells were washed once with PBS and incubated with AlexaFluor488 anti-rabbit antibody in the dark for 30min at 4°C. All cells were then washed in PBS and resuspended in 2% paraformaldehyde before analysis on a FACS Calibur analyzer (BD Biosciences, Franklin Lakes, New Jersey, USA).

siRNA transfection

Macrophages were grown in 6-well plates at a density of 1.5x106 macrophages per well for 7d, washed once with PBS, supplied with 1.5ml RMPI 1640 medium with 5% Fetal Calf Serum and 5% human AB serum (Sigma-Aldrich), and then transfected with the ON-TARGETplus GPR32 SMARTpool siRNA or non-targeting siRNA at a siRNA end concentration of 200nM for 3d (Thermo Scientific, Waltham, USA) as described previously (25). For the formation of lipid-siRNA complexes, 440µl of non-supplemented RPMI 1640 medium, 45µl of HiPerFect transfectant (Qiagen AG) and 15µl of siRNA were mixed for each 6-well vial and incubated for 20min at room temperature. 0.5ml of the mixture was then added drop-wise onto the media of adherent macrophages. After 6h, 2ml of RMPI 1640 medium with 5% Fetal Calf Serum and 5% human AB serum was added to each well.

Ca^{2+} influx assay

Macrophages were grown in 24-well plates for 7d and loaded with the calcium indicator Fluo-4 using the Fluo-4 Direct Calcium Assay Kit (Life Technologies) according to the

manufacturer instructions. The Ca2+ mobilization was monitored with a Tecan plate reader (Tecan, Maennedorf, Switzerland) measuring fluorescence (absorption 485nm, emission 520nm) every 10s for 5min. After 30s 10nM RvD1 was added to the wells. 5μM Ca2+ ionophore A-23187 (Life Technologies) was used as a positive control. Fluorescence of the Ca2+ influx assay was adjusted for cell number in each well using DAPI staining (absorption 360nm, emission 460nm).

Chemotaxis assay

A total of 5x104 macrophages were placed on a 96-well membrane (5.7mm diameter, 5μm pore size; ChemoTX from NeuroProbe, Inc., Gaithersburg, MD, USA) in RPMI 1640 containing 0.1% BSA (Sigma-Aldrich). The cells were allowed to migrate towards fMLF, MCP-1 and chemerin for 60min at the indicated concentrations. Migrated cells were fixed (2% paraformaldehyde) and stained with DAPI (Sigma-Aldrich), and migration was quantified as the total pixel count of DAPI-stained nuclei under the fluorescence microscope (two photos per membrane and three replicate wells per treatment). Migration indices were calculated over control values.

Multiplex ELISA

IL-1β, IL-1ra, IL-6, IL-8, IL-10, IL-12, GM-CSF, MCP-1, RANTES and TNFα concentrations in supernatants of primary human macrophages were measured using commercially available Bio-Plex Pro human cytokine ELISA (Bio-Rad, Hercules, USA) according to the manufacturer's instructions.

Phagocytosis assay

After stimulation with RvD1 or vehicle, cells were incubated for 30min with FITC-labeled zymosan (Life Technologies) (5 particles/cell) at 37oC with 5% CO2. After washing the cells with PBS, fluorescence was quenched using Trypan Blue (Sigma-Aldrich) (1:10 diluted) and phagocytosis was assessed using a Tecan plate reader (absorption 485nm, emission 520nm). Fluorescence of the phagocytosis assay was adjusted for cell number in each well using DAPI staining (absorption 360nm, emission 460nm).

Statistical analysis

GPR32 expression levels and functional assays were analyzed with ANOVA using Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA). $p<0.05$ was considered significant.

2.2.4 Results

GRP32 is expressed in resting, M1 and M2 primary human macrophages

To asses the expression of the RvD1 receptor GPR32 in primary human macrophages we measured mRNA and surface protein levels as we have previously done for other G protein-coupled receptors triggered by lipid mediators (21, 24, 26). GPR32 is transcribed and expressed on the surface of resting macrophages (Figure 1) and stimulation with the classical M1 polarization stimuli IFNγ and LPS, as well as with the M2 polarizing cytokines IL-4 and IL-13 did not alter mRNA transcription (Figure 1A) or surface protein expression (Figure 1B, C). Similarly, stimulation with IL-1β, TNFα and the TLR ligands CpG, poly I:C, 3M001 and 3M002 also did not affect GPR32 mRNA transcription (Figure 1A). In contrast, stimulation of macrophages with IL-6 and TGFβ reduced both, GPR32 transcription and surface expression, indicating that resting, M1, and M2 polarized macrophages show GPR32 expression on their surface, while macrophages stimulated with IL-6 or TGFβ do not.

Short term treatment of resting primary human macrophages with 10nM RvD1 induces intracellular calcium release, reduces chemotaxis and increases phagocytosis of microbial particles

To investigate whether RvD1 induces functional effects on resting primary human macrophages we treated 7d old macrophages with 1, 10, and 100nM RvD1 and measured intracellular calcium release, chemotaxis and phagocytosis. 10nM RvD1 mediated an immediate cytoplasmic calcium release in human macrophages measured by a rise in relative fluorescence shortly after RvD1 stimulation (Figure 2A). In addition, short-term exposure of macrophages for 15min with 10nM RvD1 blocked migration with maximal efficacy towards different well known macrophage chemoattractants, namely the ChemR23 ligand chemerin, the formylated peptide fMLF and MCP-1 (Figure 2B). The same RvD1 concentration increased macrophage phagocytosis of microbial particles (Figure 2C), indicating that 10nM RvD1 induces potent functional effects in resting primary human macrophages.

RvD1 effects on primary human macrophages are GPR32 dependent

It has been shown that RvD1 not only triggers GPR32 but also triggers the FPR2/ALX receptor (19). Since we have previously shown that the FPR2/ALX receptor is not expressed on the membrane of primary human macrophages (21), we investigated whether the RvD1 effects on primary human macrophages are GPR32 dependent. We therefore tested whether a reduction of GPR32 membrane expression would reduce RvD1 mediated intracellular calcium

release and RvD1 stimulated phagocytosis of microbial particles. Transient transfection of primary human macrophages with a pool of 4 siRNAs specific for GPR32 led to a significant decrease of GPR32 mRNA transcripts (Figure 3A) and ablated GPR32 surface expression after 3d (Figure 3B, C). The observed ablation of GPR32 cell surface expression by a 50% reduction in mRNA levels may be explained by the low level of basal GPR32 transcripts present in macrophages (threshold cycle 28). Macrophages lacking GPR32 did not release Ca2+ intracellularly upon 10nM RvD1 stimulation (Figure 3D). To corroborate these data, we investigated whether the increase in phagocytosis of microbial particles after 15min stimulation with RvD1 was also GPR32 dependent. Again, the stimulatory effect of 10nM RvD1 on microparticle phagocytosis by macrophages was abolished in macrophages treated with GPR32 siRNAs (Figure 3E). Similar results were obtained for macrophages stimulated with TGFβ and IL-6, which also have reduced GPR32 expression (data not shown), indicating that the RvD1 mediated effects on primary human macrophages are GPR32 dependent.

Long term RvD1 treatment of resting primary human macrophages induces a new polarization state

Our results indicate that GPR32 is present on resting macrophages and that triggering by RvD1 induces intracellular Ca2+ release, increases phagocytosis and blocks chemotaxis towards several chemoattractants. Next we investigated whether the activation of GPR32 with RvD1 would induce also a long-lasting polarization effect on resting macrophages. Stimulation for 48h with IL-4 clearly induced M2 macrophages, which secreted lower IL-1β, IL-8, MCP-1 and higher IL-1ra and displayed high CD206 on the cell surface (Figure 4). Similarly, 10nM RvD1 decreased the secretion of IL-1β, IL-8 and showed a tendency to reduce MCP-1 (Figure 4). It is noteworthy that the lower secretion of IL-1β was observed without a reduction in IL-1β mRNA levels. Such discrepancies between mRNA expression and the secretion of cytokines is in line with the post-translational regulation of IL-1β secretion (27) and seems to be a common feature also of other pro-inflammatory cytokines in resolution phase macrophages (28). RANTES, GM-CSF, IL-6 and IL-12 secretion were not changed neither by IL-4 nor by RvD1 (data not shown). However, RvD1 did not alter CD206 membrane expression characteristic for M2 macrophage polarization by IL-4. This indicates that RvD1 has the potential to polarize resting macrophages towards a pro-resolution phenotype, which is, however, different from the typical M2 polarization triggered by IL-4.

RvD1 polarized primary human macrophages migrate less towards different chemoattractant stimuli but phagocytose more microbial particles

To characterize those polarized macrophages on the functional level, we performed a chemotaxis assay towards chemerin, the formylated peptide fMLF, and MCP-1. While control human macrophages migrated towards all three chemoattractants, IL-4 polarized M2 macrophages lacked directed migration towards chemerin, the formylated peptide fMLF and MCP-1 (Figure 5A). This lack of migration was accompanied by down-regulation of the three chemokine receptors ChemR23, FPR1 and CCR2 (Supplementary Figure 1) triggered by the three chemoattractants chemerin, fMLF and MCP-1, respectively. Similarly, macrophages polarized with 10nM RvD1 did not migrate towards any of the tested chemokines (Figure 5A). However, such RvD1 stimulated macrophages still expressed high mRNA levels of ChemR23, FPR1 and CCR2 (Supplementary Figure 1), arguing for a direct role of GPR32 mediated RvD1 signaling in the regulation of actin polymerization.

In the second functional read-out, we investigated the effect of RvD1 triggered macrophage polarization on phagocytosis of microbial particles (Figure 5B). Polarization of primary human macrophages with 10nM RvD1 led to a similar increase in uptake of zymosan particles as seen after IL-4 stimulation, again supporting a role for RvD1 in polarization of macrophages towards a pro-resolution phenotype.

RvD1 has similar re- polarizing effects on primary human M1 macrophages

Since resolvins appear naturally during the resolution phase of inflammation (29), we investigated whether RvD1 causes a re-polarization of inflammatory M1 macrophages towards a pro-resolution macrophage. We therefore sequentially stimulated primary human macrophages for 3d with LPS followed by the stimulation for 4d with 10nM RvD1 and characterized the polarization phenotype of these macrophages (Supplementary Figure 2). As expected, LPS stimulated M1 macrophages exhibited an increased transcription of IL-1β, TNFα and cell surface expression of CD80 and CD206, which returned to levels comparable to untreated macrophages upon removal of the stimulus for 4d (data not shown). Sequential stimulation of these M1 macrophages with 10nM RvD1 for 4d further reduced IL-8, IL-1β and MCP-1 secretion (Supplementary Figure 2G, J, K). On the functional level RvD1 reduced chemotaxis and increased phagocytosis of microbial particles (Supplementary Figure 2L, M) indicating that re-polarized human M1 macrophages functionally resemble the macrophages observed in the RvD1 polarization experiments, displaying a pro-resolution phenotype.

2.2.5 Discussion

Here we show that the potent lipid mediator RvD1 polarizes resting primary human macrophages and re-polarizes M1 macrophages to pro-resolution type macrophages by triggering GPR32. Macrophages are a very heterogeneous group of cells which play a fundamental role during the different stages of inflammation and can be characterized by their polarization state (30). The initiation and sustaining of inflammation is mainly associated with the classically activated M1 macrophages which secrete high levels of inflammatory cytokines and produce high levels of reactive oxygen species (3), while polarization of macrophages toward a M2-like phenotype seems essential for the resolution of inflammation (31). Such alternatively activated M2 macrophages play an important role in wound healing and tissue repair (4).

However, this classification is a simplification of the polarisation states undergone by macrophages and represents the two extremes of a continuum in a universe of activation states (32). In clinical conditions, coexistence of cells in different activation states and unique or mixed phenotypes have been observed as a reflection of dynamic changes and complex tissue-derived signals (4). One of these macrophages is the resolution phase macrophage for which there is in vivo evidence for its presence during the resolution phase of inflammation in mouse models (6, 28, 33, 34). Those isolated resolution phase macrophages secrete low levels of pro-inflammatory cytokines, high levels of the anti-inflammatory IL-10 and express high levels of the mannose receptor CD206 on their surface, all proprieties of M2 polarization (6). On the other hand ciclooxygenase-2 (COX-2) expression was elevated in these resolution phase macrophages similar to M1 macrophages, indicating that resolution phase macrophages have a mixed phenotype (6). Transcriptomic analysis of resolution phase macrophages showed high levels of IL-10, COX-2 and CD206, and despite the low secretion, also high levels of inflammatory cytokines (28). In the same mouse model, peritoneal injection of RvD1 induced the formation of CD11blow macrophages with high phagocytic activity, reduced TNFα and increased IL-10 secretion reflecting the pro-resolution effect of RvD1 on mouse macrophages (33). Similarly, RvD1 treatment of obese mice lowered adipose tissue inflammation, accompanied by a re-polarization of adipose tissue M1 macrophages to a pro-resolution macrophage with lower pro-inflammatory cytokines, higher arginase 1 and higher phagocytic activity (34).

Even though some characteristics of the resolution phase mouse macrophages described in these studies differ from the characteristics of the RvD1 polarized and re-polarized primary

human macrophages in our in vitro experiments, we also observed a decrease in pro-inflammatory cytokine secretion, namely IL-1β, IL-8 and MCP-1 and an increased phagocytic capacity following RvD1 treatment, suggesting that RvD1 does indeed polarize primary human macrophages towards a resolution phase phenotype.

Differences in receptor expression and distribution as well as the experimental set-up may account for the incongruencies between the in vivo mouse studies and our in vitro human study. While RvD1 acts through the FPR2/ALX receptor in mice affecting multiple cell types following systemic application, RvD1 acts through GPR32 on macrophages only in our in vitro study on isolated primary human macrophages. Indeed, the major difference between mice and men in this context is the lack of a human GPR32 orthologue in mouse. From sequence analyses between different species it is thought that only chimpanzees have an orthologue of the human GPR32 receptor (19). In contrast, orthologues for the second RvD1 receptor, FPR2/ALX, have been identified in mouse (35) and rats (36). Hence, all the pro-resolution effects of RvD1 in mouse models depend on triggering of the FPR2/ALX receptor (20, 37, 38), while in humans both receptors play a role depending on the cell type.

We have previously shown that the FPR2/ALX receptor is absent in primary human macrophages (21), suggesting that LXA4 pro-resolution actions may be mediated by other receptors in these cells (39, 40), and we show here that RvD1 signaling in human macrophages is mediated by GPR32 involving calcium release from intracellular stores. In contrast RvD1 signaling in human neutrophils differs from the one of macrophages. Human neutrophils have been shown to mobilize FPR2/ALX and not GPR32 to the cell membrane from secretory granules upon stimulation with RvD1 (20) and signaling in neutrophils does not involve calcium release from intracellular stores (19). This argues for an elaborate cell-type specific regulation of RvD1 effects through usage of two G-protein coupled receptors with different sensitivities towards RvD1 (20) and with divergent signaling pathways for the fine tuning of the pro-resolution effects of RvD1 in human inflammation.

In spite of the different receptor usage between the two species, our data in humans are in line with the pro-resolution effects mediated by RvD1 in mouse models of inflammation. In these mouse models, RvD1 was shown to reduce neutrophil and macrophage extravasation into the inflamed area and to increase clearance of debris and apoptotic cells by macrophages (9, 19, 33). This goes along with the observed effects of RvD1 on primary human macrophages, namely the reduction in IL-8 and MCP-1 secretion, the reduced chemotaxis of these macrophages towards chemerin, fMLF and MCP-1, and the increased phagocytic activity.

In humans low nanomolar RvD1 seems to trigger two different mechanisms leading to reduced extravasation of neutrophils. On the one hand, there is evidence that RvD1 directly reduces human neutrophil migration towards IL-8 (41, 42), and on the other hand, we now show that RvD1 treated macrophages secrete less IL-8. Such a combined effect on the chemotactic activity of neutrophils and on the secretion of the chemotactic agent by macrophages may well be an efficient mechanism to block neutrophil immigration into the inflamed area.

In addition, a dual effect of RvD1 seems also to modulate human monocyte and macrophage immigration. While RvD1 treatment of human macrophages lowers the secretion of MCP-1, RvD1 treatment also directly inhibits the chemotactic migration of macrophages towards several potent chemoattractants. These in-vitro pro-resolution effects of RvD1 on primary human macrophages match the time-point at which this potent lipid mediator is found in inflammatory exudates, namely the later stages of the resolution of inflammation, where further recruitment of immune cells may not be necessary (10). In contrast LXA4, which is produced earlier during the resolution time-line, recruits monocytes via the FPR2/ALX receptor (21), and primes these monocytes towards a pro-resolution macrophage phenotype (21, 43). After these initial pro-resolution effects mediated by LXA4, it seems reasonable that the sequential rise in RvD1 during the later stages of the resolution of inflammation would further promote the polarization of macrophages towards a pro-resolution phenotype with increased phagocytic activity, however, without recruiting more leukocytes. These opposite effects of LXA4 and RvD1 on mononuclear cell recruitment also suggest a distinct therapeutic use for these two mediators, with a preferential triggering of GPR32 by RvD1 mimetics in diseases where monocyte and macrophage immigration may not be beneficial, such as in atherosclerosis.

In summary, the findings of this study provide evidence that RvD1 induces a functional switch in isolated primary human macrophages towards a pro-resolution phenotype with blocked chemotaxis, reduced secretion of inflammatory cytokines and chemokines, and increased phagocytosis of microbial particles. We further show that these actions are mediated through GPR32 triggering intracellular calcium release, highlighting its key role and possible therapeutic use for the resolution of inflammation in diseases where macrophage immigration may not be beneficial.

2.2.6 References

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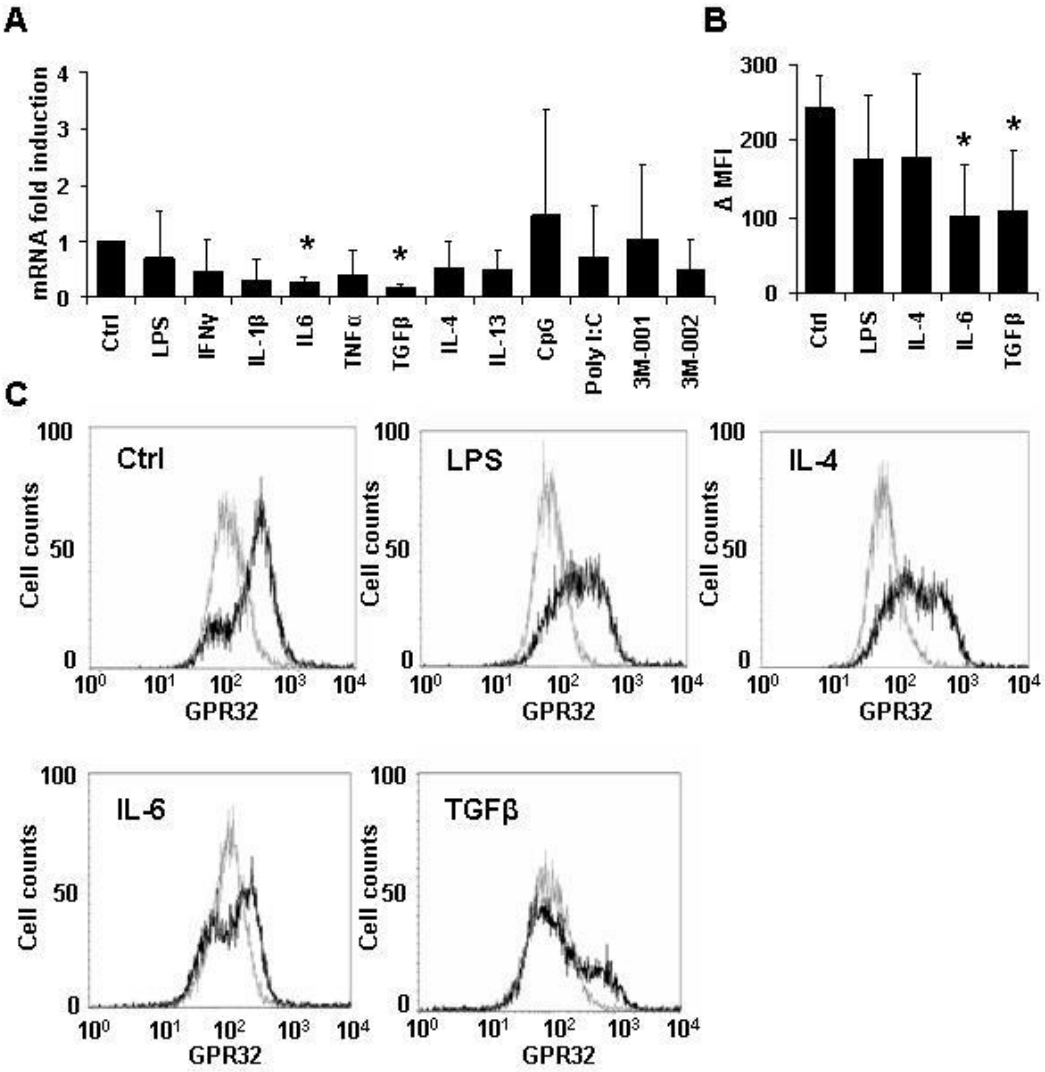
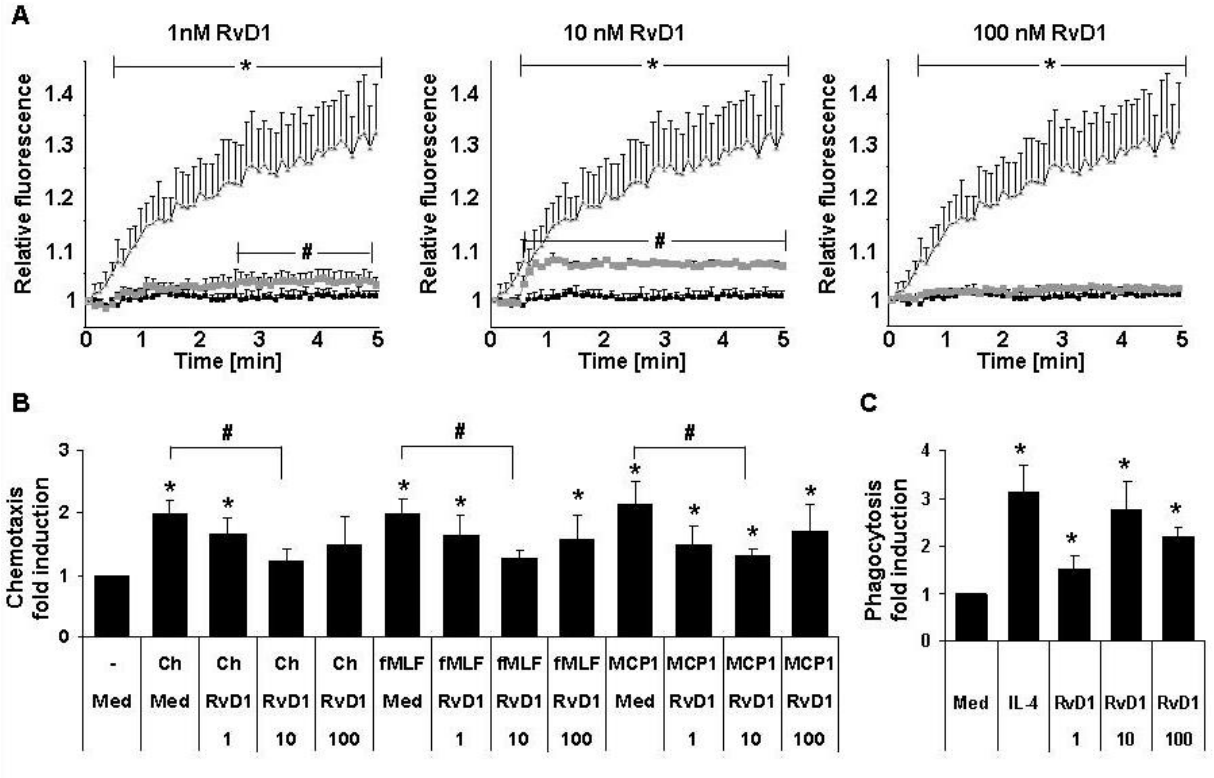
2.2.7 Figures

Figure 1: GPR32 expression on primary human macrophages



A) Relative mRNA expression of GPR32 measured by Q-PCR after stimulation of primary human macrophages with different stimuli for 24h (LPS 100ng/ml, IFN-γ 50ng/ml, IL-1β 10ng/ml, IL-6 10ng/ml, TNFα 25ng/ml, TGFβ 1ng/ml, IL-4 10ng/ml, IL-13 10ng/ml, CpG 100ng/ml, Poly I:C 1ng/ml, 3M001 3µM, 3M002 3µM). All values are normalized for GAPDH and are presented relative to unstimulated macrophages. Bars indicate the mean and SD of 3 independent experiments. B) GPR32 protein expression on macrophages stimulated for 48h measured by FACS. Values are presented as the difference between the median fluorescence intensity of the GPR32 Ab and the isotype control. Bar indicates the mean and SD of 3 independent experiments. C) Representative graphs of the FACS results. The isotype control is shown in gray and cells labelled with GPR32 Ab in black. *$p<0.05$.

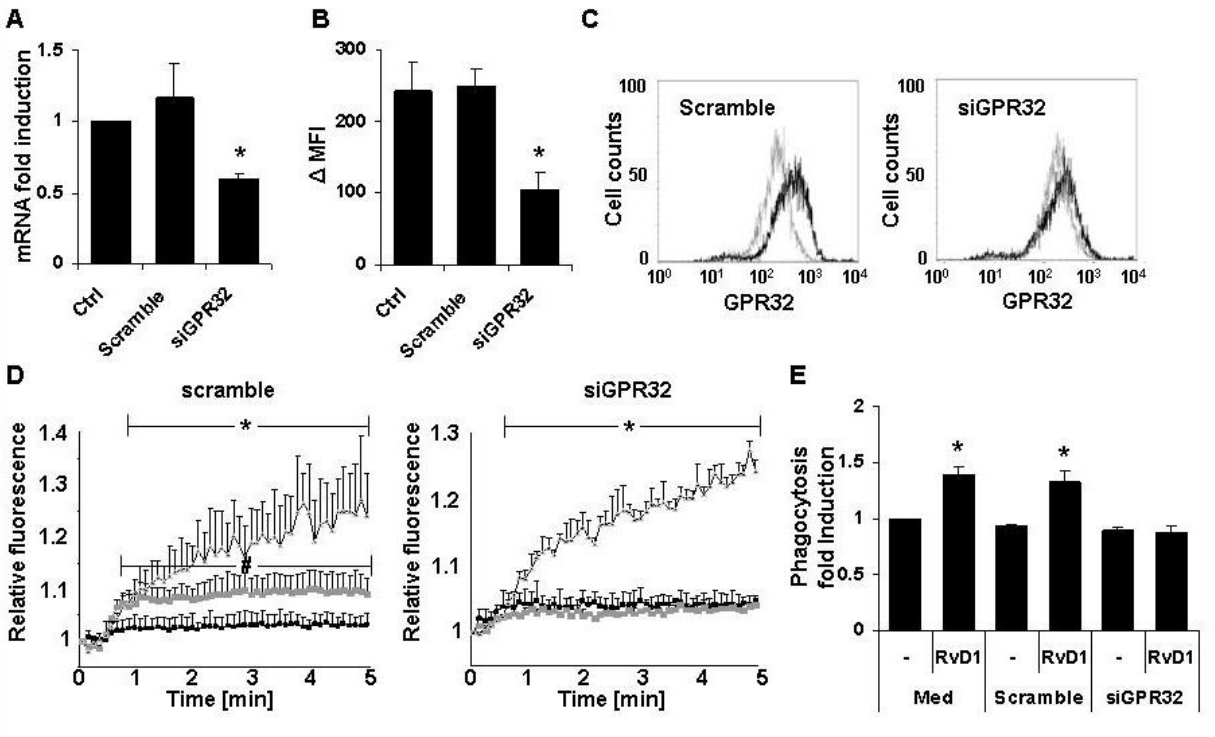
Figure 2: RvD1 induces dose-dependent short term functional changes in primary human macrophages



RvD1 triggers intracellular $Ca2^{+}$ release, blocks chemotactic migration and stimulates phagocytosis of microbial particles with maximal efficiency at 10nM. A) Ca^{2+} monitoring using Fluo-4 as an indicator. 30s after the start of the experiment 1, 10 or 100nM RvD1 (gray squares), or 5µM Ca^{2+} ionophore A-23187 (gray line) were given to human macrophages. Non-stimulated control cells are shown in black squares. The fluorescence levels are presented relative to the starting value. The symbols indicate the mean and upper bars represent the SD of 3 independent experiments. *$p<0.05$ for comparison between cells with no stimuli (black squares) and cells stimulated with A-23187 (gray line). $^{\#}p<0.05$ for comparison between cells with no stimuli (black squares) and cells stimulated with RvD1 (grey squares). In panels B and C resting macrophages were stimulated for 15min in the presence of medium (Med), 1, 10 or 100nM RvD1, and chemotactic migration or phagocytosis of zymosam particles was measured. B) Chemotaxis of macrophages towards 10nM chemerin (Ch), 10nM formylated peptide (fMLF) and 100ng/ml MCP-1 was measured. Bars indicate the mean and SD of 3 independent experiments. *$p<0.05$ compared to basal migration of macrophages without stimuli. $^{\#}p<0.05$ compared to chemotaxis toward Ch, fMLF or MCP-1 without RvD1 stimulation. C) Phagocytosis of zymosan particles was

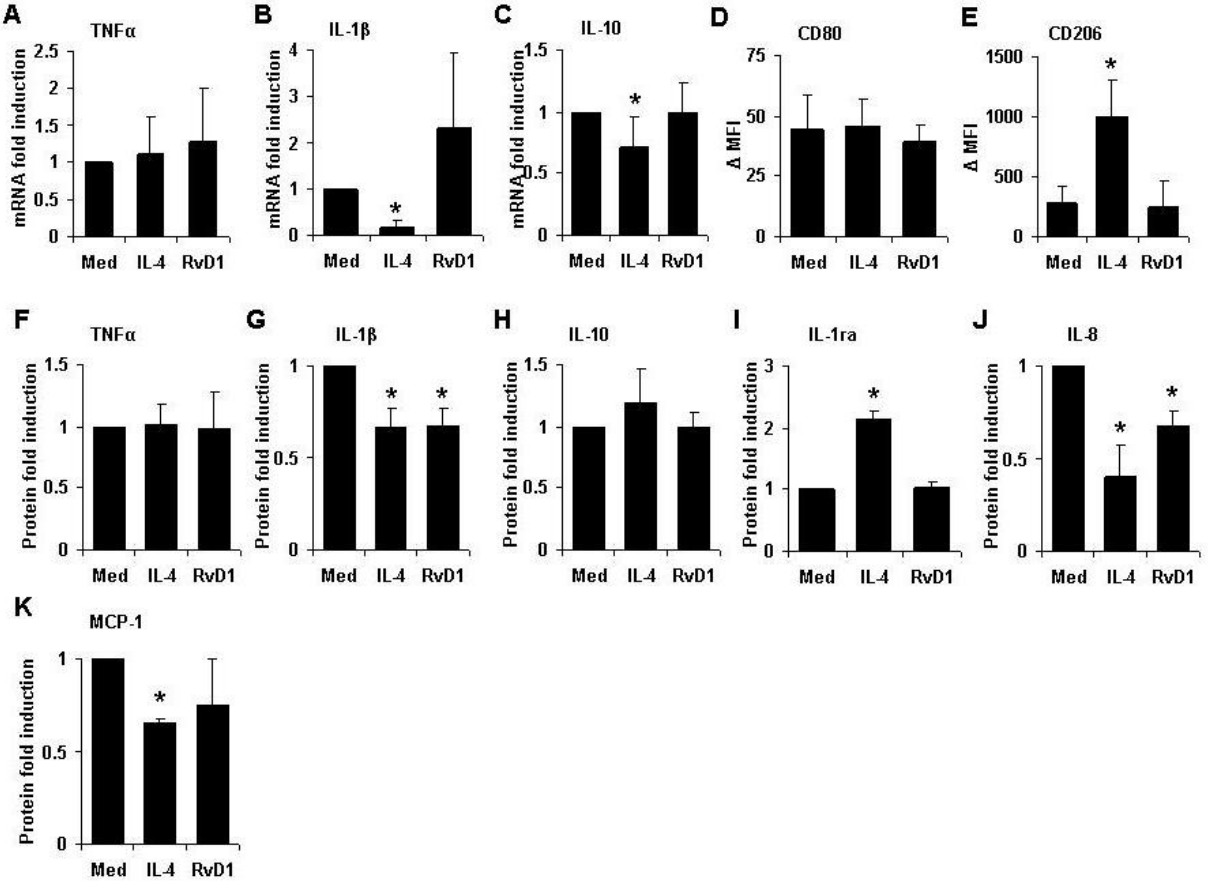
measured. Values are presented relative to unstimulated macrophages. Bars indicate the mean and SD of 3 independent experiments. *$p<0.05$.

Figure 3: RvD1 effects on primary human macrophages are GPR32 dependent



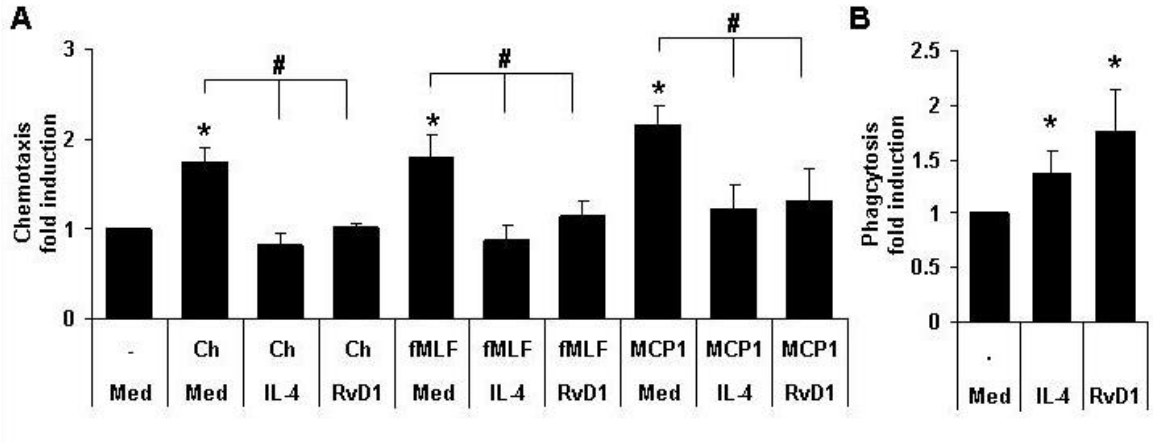
Human primary macrophages transfected with specific siRNA for GPR32 (siGPR32) or non-targeting siRNA (Scramble) were tested for RvD1 responsiveness. A) Relative mRNA expression of GPR32 measured with Q-PCR after transfection of primary human macrophages with 200nM siRNA for 72h. All values are normalized for GAPDH and are presented relative to non-transfected macrophages. Bar indicates the means and SD of 3 independent experiments. B) GPR32 protein expression on macrophages by FACS. Values are presented as the difference between the median fluorescence intensity of the GPR32 Ab and the isotype control. Bars indicate the mean and SD of 3 independent experiments. C) Representative graphs of the FACS results. The isotype control is shown in gray and cells labeled with GPR32 Ab in black. D) Ca^{2+} monitoring using Fluo-4 as an indicator. 30s after the start of the experiment, 10nM RvD1 (gray squares), or 5μM Ca^{2+} ionophore A-23187 (gray line) were given to human macrophages. Non-stimulated control cells are shown in black squares. The fluorescence levels are presented relative to the starting values. The symbols indicate the means, and upper bars represent the SD of 3 independent experiments. *$p<0.05$ for comparison between cells with no stimuli (black squares) and cells stimulated with A-23187 (gray line). $^{\#}p<0.05$ for comparison between cells with no stimuli (black squares) and cells stimulated with 10nM RvD1 (gray squares). E) Phagocytosis of zymosan particles was measured. Values are presented relative to unstimulated macrophages. Bars indicate the mean and SD of 3 independent experiments. *$p<0.05$.

Figure 4: RvD1 polarizes resting primary human macrophages to a new pro-resolution phenotype



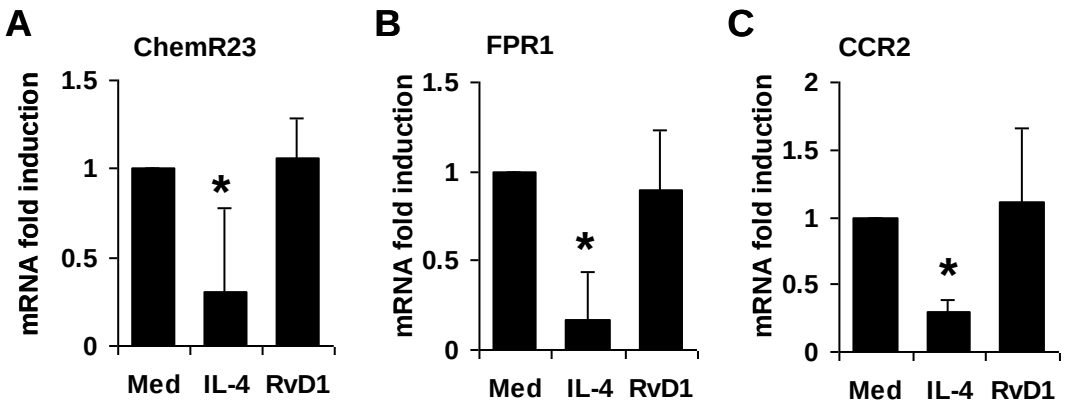
Resting primary human macrophages were polarized for 48h in the presence of medium (Med), IL-4 or 10nM RvD1. mRNA and protein expression of cytokines and cell surface markers were measured to characterize polarization of macrophages. In the panels A-C mRNA levels of A) TNFα, B) IL-1β, and C) IL-10 measured by Q-PCR are presented. All mRNA values are normalized for GAPDH and are presented relative to macrophages polarized with medium alone (Med). In panels D and E membrane expression of D) CD80, and E) CD206 measured by FACS analysis is shown, with values presented as the difference between the median fluorescence intensity of the Abs and their respective isotype controls. In panels F-K protein secretion of F) TNFα, G) IL-1β, H) IL-10, I) IL-1ra, J) IL-8, and K) MCP-1 measured with a multiplex ELISA in the supernatants is shown. Values are presented relative to unstimulated macrophages. Bars indicate the mean and SD of 3 independent experiments. *$p<0.05$.

Figure 5: RvD1 polarized macrophages are less chemotactic and phagocytose more microbial particles



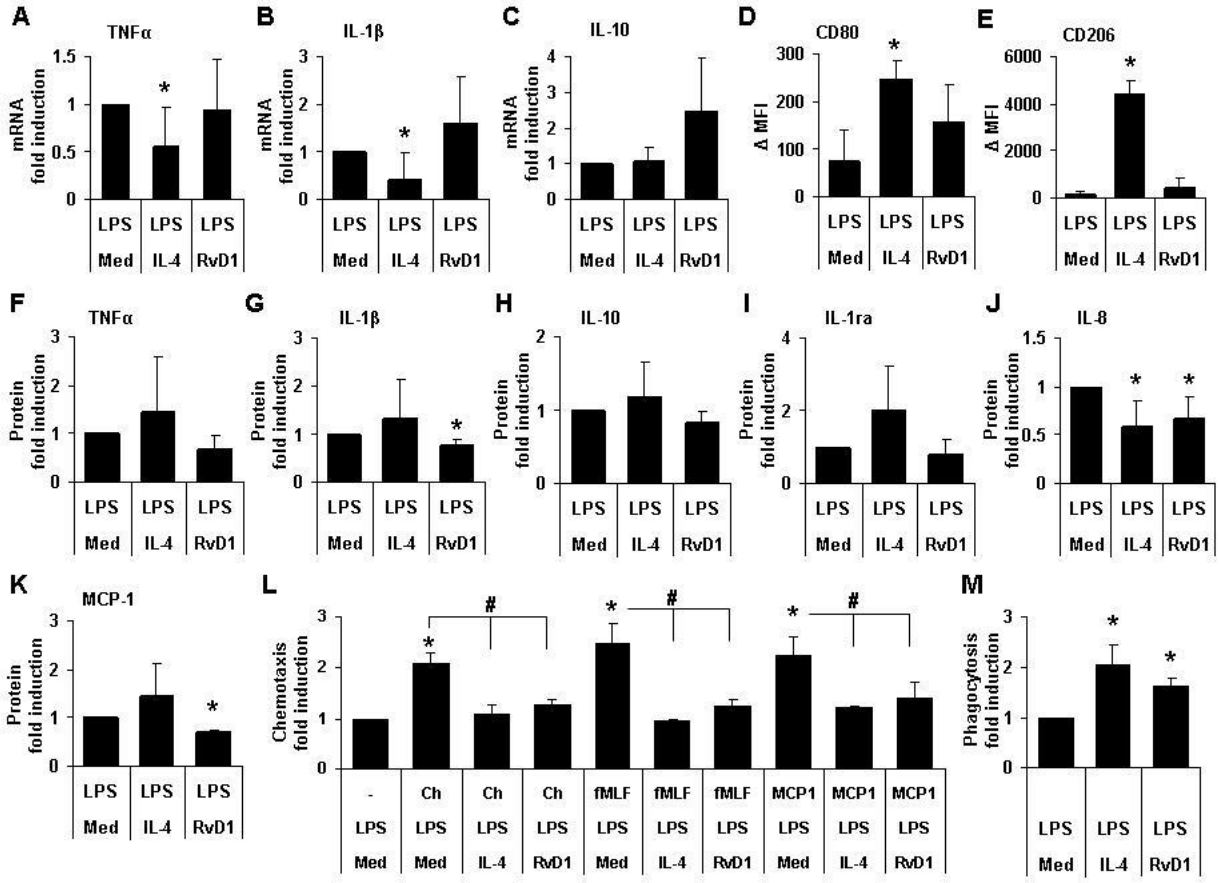
Resting primary human macrophages were polarized for 48h in the presence of medium (Med), IL-4 or 10nM RvD1 and afterwards tested for chemotactic migration and phagocytosis. A) Chemotactic migration of macrophages towards 10nM chemerin (Ch), 10nM formylated peptide (fMLF) and 100ng/ml MCP-1. Values are presented relative to the basal migration of macrophages without stimuli. Bars indicate the mean and SD of 3 independent experiments. *$p<0.05$ compared to basal migration of macrophages without stimuli. $^{\#}p<0.05$ compared to chemotaxis towards Ch, fMLF or MCP-1 without IL-4 or RvD1 stimulation. B) Phagocytosis of zymosan particles. Values are presented relative to unstimulated macrophages. Bars indicate the mean and SD of 3 independent experiments. *$p<0.05$.

Supplementary Figure 1: IL-4 reduces chemokine receptor expression, while RvD1 does not



Resting primary human macrophages were polarized for 48h in the presence of medium (Med), IL-4 or 10nM RvD1 and afterwards tested for mRNA expression of chemokine receptors. A) ChemR23, B) FPR1, and C) CCR2 mRNA levels measured by Q-PCR are presented. All mRNA values are normalized for GAPDH and are presented relative to macrophages polarized with medium alone (Med). Bars indicate the mean and SD of 3 independent experiments. *$p<0.05$.

Supplementary Figure 2: RvD1 re-polarizes M1 primary human macrophages to a pro-resolution phenotype



Resting primary human macrophages were polarized toward an M1 phenotype with 100ng/ml LPS for 3d. These M1 macrophages were then re-polarized for 4d in the presence of medium (Med), IL-4 or 10nM RvD1. mRNA and protein expression of cytokines, cell surface markers, chemotaxis and phagocytosis were measured to characterize the re-polarization of macrophages. In panels A-C mRNA levels of A) TNFα, B) IL-1β, and C) IL-10 measured by Q-PCR are presented. All mRNA values are normalized for GAPDH and are presented relative to M1 macrophages re-polarized with medium alone (Med). In panels D and E membrane expression of D) CD80, and E) CD206 measured by FACS analysis is shown. Values are presented as the difference between the median fluorescence intensity of the Abs and their respective isotype controls. In panels F-K protein secretion of F) TNFα, G) IL-1β, H) IL-10, I) IL-1ra, J) IL-8, and K) MCP-1 measured with a multiplex ELISA in supernatants is shown. Bars indicate the mean and SD of 3 independent experiments. *$p<0.05$. L) Chemotactic migration of macrophages towards 10nM chemerin (Ch), 10nM formylated peptide (fMLF) and 100ng/ml MCP-1. Values are presented relative to the basal migration of macrophages without stimuli. Bars indicate the mean and SD of 3 independent experiments.

*p<0.05 compared to basal migration of M1 macrophages re-polarized with medium alone without stimuli. #p<0.05 compared to chemotaxis towards Ch, fMLF or MCP-1 without IL-4 or RvD1 stimulation. M) Phagocytosis of zymosan particles. Values are presented relative to M1 macrophages re-polarized with medium alone. Bars indicate the mean and SD of 3 independent experiments. *p<0.05.

3. Conclusions

Current model of inflammation and its resolution

Combining the recent publications of our research group [27, 216-218] and the literature, we postulate a mechanistic model of initiation and resolution of acute inflammation in humans (Figure 7). When pathogens invade the tissue, PAMPs and DAMPs released by damaged cells are sensed by resident APC cells, mostly macrophages and dendritic cells. Those cells create a chemotactic milieu which favors the recruitment of the pro-inflammatory neutrophils. Of notable importance is the lipid mediator LTB4, which is produced by macrophages [219] and binds to the neutrophilic cell surface receptor BLT1 [62], the cytokine IL-8 produced by macrophages [220] and activated endothelial cells [221], the complement system derived anaphylatoxins C3a, C4a and C5a whose physiological functions include an increase in vascular permeability, leukocyte recruitment and muscle cell contraction [222], and the formylated peptides which are released by bacterial membranes or damaged mitochondria and bind to the FPR1 receptor [81, 195, 223]. This activated environment also promotes the development of classically activated M1 macrophages in the tissue, which help neutrophils to eliminate the noxious agents by producing high levels of ROS, RNS, and by secreting high levels of TNFα, IL-1β, IL-8 and MCP-1 [35, 224]. TNFα and IL-1β increase the vascular inflammation while IL-8 recruits further neutrophils into the inflamed tissue. The high levels of MCP-1, formylated peptides, and the neutrophil-mediated maturation of chemerin [225] contribute to the recruitment of more classically activated M1 macrophages, which express high level of the CCR2 [226], FPR1 [216] and ChemR23 [217] receptors, necessary for chemotaxis by these peptides, respectively.

The above described pro-inflammatory cascade is essential to contain and eliminate the invading pathogens, but needs to be stopped once the insulting agents have been removed. The resolution of inflammation is characterized by two key cellular events: a reduction in neutrophil infiltration and a re-polarization of macrophages from the pro-inflammatory M1 phenotype to a M2-like phenotype [227]. These resolution phase macrophages secrete lower levels of pro-inflammatory cytokines and display proficient phagocytosis to clear apoptotic neutrophils and debris form the tissue [39].

While M1 macrophages express an array of chemotactic receptors which guide them to the affected loci, pro-resolution macrophages lose their chemotactic ability. On the same line, M2 macrophages stimulated with IL-4 or IL-13 down-regulated the expression of FPR1 and were unresponsive to fMLF [216]. This link between macrophage phenotype and FPR1 expression,

underlines one of the mechanisms by which macrophage re-polarization can regulate chemotaxis and initiate the resolution program of inflammation.

In addition to the repolarisation of macrophages by Th2 cytokines, LXA4, RvE1 and RvD1 play an essential role in driving the resolution of inflammation by controlling macrophage polarization and reducing neutrophil chemotaxis. In the first place all these lipid mediators act directly on neutrophils: LXA4 and RvE1 block chemotaxis antagonizing LTB4 at the BLT1 receptor level [228], while RvD1 regulates actin polymerization via the FPR2/ALX receptor [58].

The other main effects of these lipid mediators during the course of inflammation are seen on macrophage polarization. During the inflammatory phase, the elevated production of the prostaglandins induces the transcription of 15LOX, which switches the synthesis of lipid mediators from LTB4 towards LXA4 [122]. This omega-6 derived lipid mediator acts though the FPR2/ALX receptor, which is present on monocytes but not on macrophages [27].We and others could show that LXA4 attracts monocytes [26, 27] and primes them toward a pro-resolution phenotype macrophage which is able to take up debris and apoptotic cells from the inflamed tissue [113], initiating resolution of inflammation. In a later resolution phase, when further mononuclear cell recruitment may not be needed anymore, RvE1 blocks chemerin mediated chemotaxis of macrophages via the ChemR23 receptor [217]. The activated M1 macrophages expressing high levels of ChemR23 are also ready to be re-polarized by RvE1 to a pro-resolution phenotype, which looses not only its chemotaxis towards chemerin but also has a higher phagocytic capacity and a higher transcription of the anti-inflammatory IL-10 [217]. Notably M2 macrophages have low ChemR23, indicating that RvE1 acts as a trigger for initiation of pro-resolution programs in M1 macrophages and has less impact, once the re-polarization process has been initiated [217]. After the priming of monocytes by LXA4 and the re-polarization of macrophages by RvE1, RvD1 acts on all kind of macrophages ensuring the resolution of inflammation. In fact we have shown that RvD1 acts through GPR32 in resting and M1 macrophages to induce a pro-resolution phenotype, which is not chemotactic anymore to different stimuli, secretes less pro-inflammatory cytokines and displays higher phagocytosis.

In conclusion, LXA4, RvE1 and RvD1 are potent pro-resolution lipid mediators with partly overlapping and partly distinct actions, which act in a chronological manner to orchestrate the resolution of inflammation in humans.

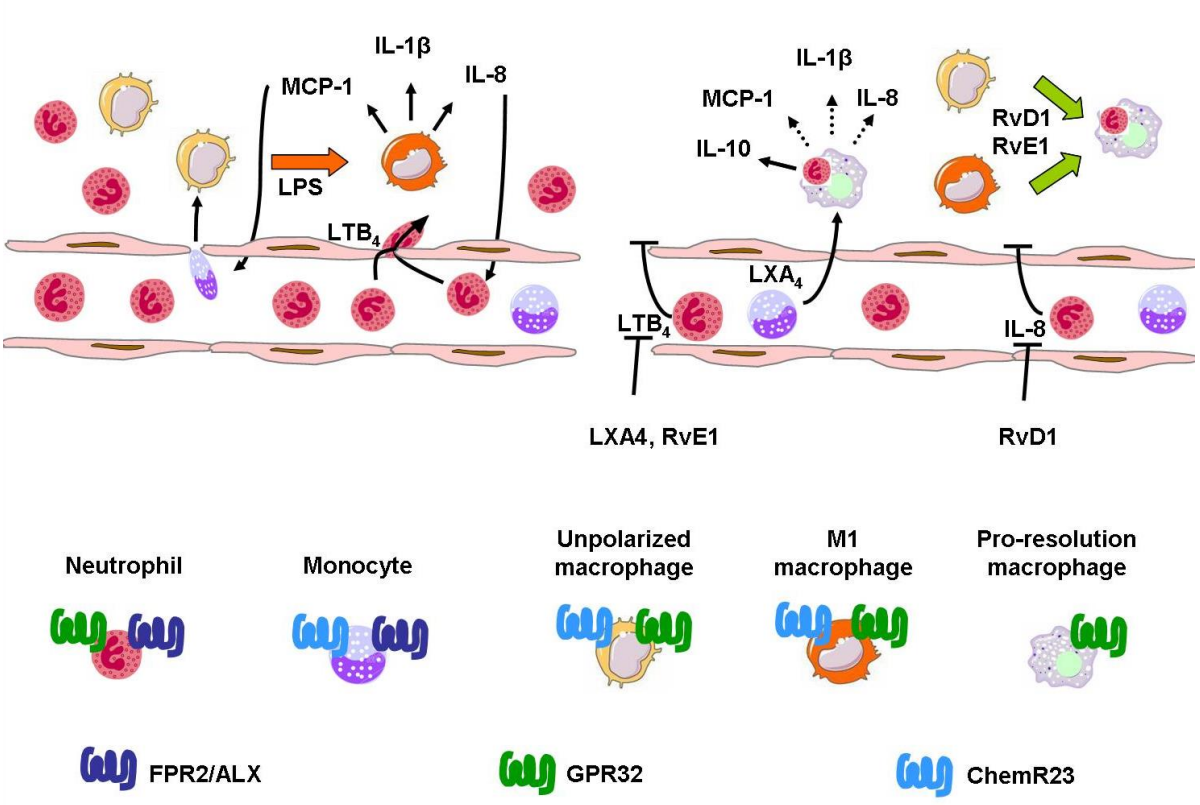


Figure 7: Resolution of inflammation is mediated by the chronological action of LXA4, RvE1 and RvD1

Pathogen invasion or tissue injury induces tissue resident APC to release mediators such as LTB4 and IL-8 which recruits neutrophils. This induces a cascade of pro-inflammatory events that promote M1 macrophages and further recruitment of leukocytes. LXA4 is the first mediator produced which blocks neutrophil chemotaxis and recruits non-phlogistic monocytes that will develop to pro-resolution macrophages. Resolvins appear later in the resolution of inflammation and further reduce the infiltration of neutrophils and promote the re-polarization of macrophages toward a pro-resolution phenotype which secrete less chemoattractants and take up apoptotic cells and debris.

The pro-resolution effects of RvD1 on primary human macrophages

A peculiar effect of RvD1 compared to other pro-resolution lipid mediators is the inhibition of macrophage chemotaxis toward different stimuli, namely the peptide chemerin, the formylated peptide fMLF, and MCP-1. In contrast, RvE1 can only block chemerin mediated cell migration [217] by directly antagonizing the interaction of chemerin with the ChemR23 receptor [57]. This is probably not the case for RvD1, since antagonism of three different receptors seems improbable. More likely RvD1 binds GPR32 on human macrophages and regulates actin polymerization to block chemotaxis, in a similar way to what happens in RvD1 stimulated neutrophils [58]. In neutrophils actin regulation was pertussis toxin sensitive, indicating that G protein activation is the underlying mechanism [58].

Activation of the Gαi family of G proteins is usually required for chemotaxis, inducing a signalling cascade which involves the production of PIP3 and cytoskeleton reorganization [67]. This is for example the case of the FPR2/ALX receptor, which when activated by aspirin triggered lipoxins, releases the active Gαi, inducing the Rho/Rho kinase pathway and the cytoskeletal reorganisation needed for monocyte chemotaxis [229].

The two classes of G-proteins Gαi and Gαs have opposing signalling pathways, with Gαi inhibiting the formation of cAMP, while Gαs promotes it. Therefore a possible explanation for the inhibiton of macrophage chemotaxis by triggering GPR32 with RvD1 would be a signalling cascade involving Gαs. This is however only one possibility among the complex signalling cascade of GPCRs [230]. In fact, until recently, only Gαi was thought to be implicated in leukocyte chemotaxis, however it is now clear that also Gαq/11 is able to regulate cell migration [231], indicating new possible regulatory pathways by which RvD1 could inhibit chemotaxis.

Nevertheless, the inhibition of macrophage chemotaxis towards different stimuli, which is a specific propriety of RvD1, combined with the reduced secretion of MCP-1, suggest a possible therapeutic use in the resolution of inflammation in human diseases where macrophage infiltration may not be beneficial, such as atherosclerosis.

The reduction of pro-inflammatory cytokine secretion from primary human macrophages is another important feature of RvD1. The common factor linked to the production of IL-1β, IL-8 and MCP-1 is NF-κB [232-234], which is known to be down-regulated by RvD1 signalling [58]. Recently, another group started to investigate the signalling events that lead to the suppression of NF-κB upon RvD1 stimulation [235]. In this study RvD1 largely blocked the TRAF/TAK1/TAB1 association and TAK1 phosphorylation, both needed for the nuclear translocation of NF-κB, in a β-arrestin dependent manner, indicating that RvD1 may be able to promote resolution of inflammation also by activating β-arrestin or other signalling pathways.

RvD1 mediated actions: differences between mice and man

In our study we could show that RvD1 blocks chemotaxis, lowers pro-inflammatory cytokine secretion and increases phagocytosis of primary human macrophages. These pro-resolution characteristics are comparable to what has been found in mouse studies, however, with some differences. While RvD1 treated mouse macrophages highly expressed CD206 and secreted high levels of IL-10, in our experiments we could not observe an alteration of cell surface markers following RvD1 treatment compared to M1 macrophages, as well as no increase in IL-10 production [39, 141, 157, 158].

These incongruencies can be explained by differences in receptor expression and distribution as well as by the experimental set-up. While RvD1 acts through FPR2/ALX receptor in mice affecting multiple cell types following systemic application, RvD1 acts through GPR32 on macrophages only in our in vitro study on primary human macrophages. Indeed, the major difference between mice and men in this context is the lack of a human GPR32 orthologue in mouse. From sequence analyses between different species it is thought that only chimpanzees have an orthologue of the human GPR32 receptor [58]. In contrast, orthologues for the second RvD1 receptor, FPR2/ALX, have been identified in mouse [236] and rats [208]. Hence all the pro-resolution effects of RvD1 in mouse models depend on triggering of the FPR2/ALX receptor [133, 210, 211], while in humans both receptors play a role depending on the cell type.

We have previously shown that the FPR2/ALX receptor is absent in primary human macrophages [27] and we show here that RvD1 signalling in human macrophages is mediated by GPR32, involving Ca^{2+} release from intracellular stores. On the other hand, the FPR2/ALX receptor is the main player mediating RvD1 actions in human neutrophils, since in contrary to GPR32, it gets mobilized to the cell membrane upon RvD1 stimulation [133].

Interestingly, RvD1 signalling in neutrophils through FPR2/ALX does not involve calcium release from intracellular stores [58], unlike the stimulation of GPR32 on macrophages shown here. Ca^{2+} is an important second messenger of several GPCRs which drives gene expression and many other physiological processes [237]. Ca^{2+} influx usually involves receptor-dependent activation of PLCβ mediated by the Gαq/11 class subunit or by the released Gβγ. PLCβ hydrolyses the plasma membrane lipid PIP2, to generate IP3 in the cytosol. IP3 activates the endoplasmic reticulum receptor IP3R, releasing Ca^{2+} stored in the ER into the cytoplasm [238]. Given the fact that human macrophages release Ca^{2+} upon triggering of GPR32 with RvD1, it is reasonable to propose a role for Gαq/11 signalling in human macrophages. In contrast RvD1 does not regulate Ca^{2+} (second messenger of Gαq/11) nor cAMP (second messenger of Gαs and Gαi) in human neutrophils through FPR2/ALX [58], indicating that Gα12/13 may be the key Gα signalling subunit in this case.

Taken together these results suggest that RvD1 acts on macrophages via GPR32 signalling through Gαs blocking chemotaxis and through Gαq/11 releasing Ca^{2+}. On contrary RvD1 activates FPR2/ALX on neutrophils and may signal through Gα12/13. This argues for an elaborate cell-type specific regulation of RvD1 effects through usage of two GPCRs and different signalling Gα subunits for the fine tuning of the pro-resolution effects of RvD1 in humans.

To conclude, the identification of GPR32 as the main player in the RvD1 mediated polarization of human macrophages may indicate its possible usage as a therapeutic target. On this line of thinking, very recently the first RvD1 analog mimetic binding GPR32 has been synthesized which displays potent pro-resolution actions in vitro in human cells such as a reduction of neutrophil chemotaxis and increased phagocytosis by macrophages [239]. However, because of the presence of the two receptors in other human tissues, which display different signalling pathways, the RvD1 actions may be more complex in humans than in mice, highlighting the importance of further studies on isolated human cells.

4. References

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Own contribution to publications

-Regulation of the formyl peptide receptor 1 (FPR1) gene in primary human macrophages

FACS analysis, chemotaxis assays, data evaluation.

-Resolvin D1 polarizes primary human macrophages towards a pro-resolution phenotype through GPR32

Designed and performed all experiments, data evaluation, manuscript writing.

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